IOWA STATE UNIVERSITY
Digital Repository

Retrospective Theses and Dissertations

Iowa State University Capstones, Theses and Dissertations

1989

Physiology of the wall-less Anaeroplasmataceae (Class Mollicutes) and related walled bacteria

James P. Petzel
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

 Part of the Microbiology Commons

Recommended Citation

Petzel, James P., "Physiology of the wall-less Anaeroplasmataceae (Class Mollicutes) and related walled bacteria " (1989). *Retrospective Theses and Dissertations*. 9077.
https://lib.dr.iastate.edu/rtd/9077

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.

INFORMATION TO USERS

The most advanced technology has been used to photograph and reproduce this manuscript from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book. These are also available as one exposure on a standard 35mm slide or as a 17" x 23" black and white photographic print for an additional charge.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

U·M·I

University Microfilms International
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
313/761-4700 800/521-0600

Order Number 9003557

Physiology of the wall-less *Anaeroplasmataceae* (Class *Mollicutes*) and related walled bacteria

Petzel, James P., Ph.D.

Iowa State University, 1989

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

Physiology of the wall-less *Anaeroplasmataceae* (Class *Mollicutes*) and related walled bacteria

by

James P. Petzel

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major: Microbiology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Members of the Committee:

Signature was redacted for privacy.

Iowa State University
Ames, Iowa
1989

TABLE OF CONTENTS

"We dance round in a ring and suppose
But the secret sits in the middle and knows."

The Secret Sits, by Robert Frost.

GENERAL INTRODUCTION

The wall-less bacteria of the class *Mollicutes* (formerly called mycoplasmas) are classified into three orders: the *Mycoplasmatales*, which include the sterol-requiring genera *Mycoplasma*, *Ureaplasma*, and *Spiroplasma;* the *Acholeplasmatales*, which include the sterol-nonrequiring genus *Acholeplasma;* and the *Anaeroplasmatales*, which include the sterol-requiring *Anaeroplasma* and the sterol-nonrequiring *Asteroleplasma.*

The *Mycoplasmatales* and *Acholeplasmatales* have been studied for a number of years because of their importance in human and animal medicine. In addition, Morowitz (1984) has proposed that because the mollicutes are the smallest, free-living cells with small genomes and limited metabolic capabilities, they should serve as model organisms in an investigation of minimal requirements for independent cellular life. Many aspects of the carbohydrate, purine, pyrimidine, arginine, and urea metabolism and of the membrane physiology in the *Mycoplasmatales* and *Acholeplasmatales* have been elucidated. On the other hand, the first strictly anaerobic mollicute was not described until 1973, and little is known about the intracellular metabolism of the *Anaeroplasmatales.* Therefore, I investigated some aspects of the physiology of the strictly anaerobic mollicutes.

In this dissertation, some enzymic activities associated with carbohydrate, purine, and pyrimidine metabolism were described for *Anaeroplasma intermedium* and *Asteroleplasma anaerobium.* The most

distinctive feature noted in these organisms was the presence of several pyrophosphate-dependent (PP_i-dependent) enzymes; some of these enzymes had been detected previously in other mollicutes by other investigators.

These PP_i-dependent enzymes occur infrequently among most bacteria. However, because these enzymes occur with greater frequency in the *Mollicutes*, six species of walled bacteria proposed to be phylogenetically related to the *Mollicutes* based on rRNA-sequence analyses were examined for PP_i-dependent enzymes. The demonstration of one or more PP_i-dependent enzymes in each of the walled bacteria is a general phenotypic indicator of the phylogenetic relatedness of these bacteria and the *Mollicutes*, and the distributions of the PP_i-dependent enzymes among these bacteria correlated with phylogenetic subdivisions of this group based on 16S-rRNA analysis.

Dissertation Format

This dissertation is in the alternate thesis format; it includes two sections that are manuscripts. The manuscript that forms Section I has been accepted for publication in *Archives of Microbiology*. Section II is a manuscript that has been submitted to the *International Journal of Systematic Bacteriology*. A general literature review precedes the first manuscript, and a general summary and discussion follows the second manuscript. There are separate lists of references for each of the manuscripts as well as a separate list for the literature review and summary and discussion.

The doctoral candidate, James P. Petzel, was the principal investigator in these studies with the exception of the examination of the Embden-Meyerhof-Parnas pathway in *Anaeroplasma intermedium* and pyrimidine metabolism in *Asteroleplasma anaerobium*. The data for these studies were collected by David DeSantis and Marshall V. Williams of the Ohio State University, respectively, using cells provided by the doctoral candidate. In addition, the doctoral candidate provided cells used in the investigations of McElwain et al. (1988) and Weisburg et al. (1989) and is co-author on those reports.

LITERATURE REVIEW

The Class *Mollicutes*

Introduction and history

Prokaryotes that are devoid of cell walls are grouped in the Class *Mollicutes.* These organisms are very small (0.1 to 0.3 µm in diameter), and they can pass through membrane filters that retain other bacteria. *Mollicutes* are divided into three orders, the *Mycoplasmatales* (Freundt et al., 1984; Razin and Freundt, 1984), the *Acholeplasmatales* (Freundt et al., 1984), and the *Anaeroplasmatales* (Robinson and Freundt, 1987). Unlike these eubacteria, the remaining organism in the Class *Mollicutes*, *Thermoplasma acidophilum*, is an archeobacterium (Razin and Freundt, 1984; Woese et al., 1980)., and it will not be discussed further.

The term "mollicutes" is currently used to refer to wall-less bacteria (Razin and Freundt, 1984). Previously, the term "mycoplasma" was used to refer to either any wall-less bacterium or specifically to members of the genus *Mycoplasma*, causing confusion. In the present discussion, the term "mollicutes" will be used, even when discussing publications that appeared before the term was invented. The names "mycoplasmas", "ureaplasmas", "spiroplasmas", and "acholeplasmas" will be used to refer to members of the respective genera. The term "strictly anaerobic mollicutes" will be used to refer to members of the family *Anaeroplasmataceae.* For clarity, the generic name "*Anaeroplasma*" will be abbreviated "*An.*", "*Asteroleplasma*" will be abbreviated "*As.*", and

"*Acholeplasma*" will be abbreviated "*A.*".

The first isolation of a mollicute was by Nocard and Roux in 1898 (cited by Smith, 1971), who reported that they had isolated the etiological agent of contagious bovine pleuropneumonia, which they believed was a virus. Dujardin-Beaumetz (cited by Hayflick, 1969) was the first to cultivate this mollicute on a solid medium; he described the distinctive colonial morphology of mollicutes. This morphology, often referred to as a "fried-egg" colony, consists of a dark center that penetrates the medium, surrounded by a lighter periphery. In the first half of this century, similar mollicutes were isolated from a variety of animals, and these organisms became known as "pleuropneumonia-like organisms" or "PPLO". This term was eventually supplanted by the name *Mycoplasma* (Hayflick, 1969). The agent of contagious bovine pleuropneumonia is now called *Mycoplasma mycoides* subsp. *mycoides*.

Dienes and Edsall reported the first isolation of a mollicute from a human in 1937, but it was not until 1962 that Chanock et al. provided etiologic proof of the involvement of a mollicute in a human disease, primary atypical pneumonia. Today, this organism is called *Mycoplasma pneumoniae*.

In 1936, Laidlaw and Elford isolated a mollicute from sewage that they believed was a saprophyte. Another strain of this organism was later isolated from the bovine genital tract (Edward, 1950). This and related species do not require sterols, and thus, they were placed into a separate genus, *Acholeplasma* (Edward and Freundt, 1970). It is now known

that acholeplasmas are parasites of animals and insects, as well as saprophytes of plants (Tully et al., 1988).

In 1956, Shepard described a mollicute associated with nongonococcal urethritis that produced very small colonies. First called "T-strains" and eventually named *Ureaplasma urealyticum* (Shepard et al., 1974), the pathogenic involvement of this organism in nongonoccal urethritis was confirmed by Taylor-Robinson et al. (1977).

Spiroplasmas, often associated with diseased plants, were first observed by microscopy, but they were mistaken for spirochaetes (Poulson and Sakaguchi, 1961). They were observed twice in the 1960s, but they were thought to be either a virus or a spirochaete (Whitcomb and Tully, 1984). It was not until 1973 that they were well-characterized as mollicutes (Saglio et al., 1973).

The strictly anaerobic mollicutes from bovine and ovine rumens were first noted by R. E. Hungate because of their ability to lyse Gram-negative bacteria (Hungate, 1966, 1970). One strain of these bacteria was characterized by J. P. Robinson and Hungate (1973) and assigned to the new species *Acholeplasma bactoclasticum.* I. M. Robinson and colleagues (Robinson, 1973, 1979; Robinson and Allison, 1975; Robinson et al., 1975) were also able to isolate strictly anaerobic mollicutes from the rumen, as were Prins and van der Vorstenbosch (1975). I. M. Robinson and Allison (1975) determined that *Acholeplasma bactoclasticum* was not a true acholeplasma because it utilized sterols obtained from Gram-negative cells added to the medium. Because the organism failed to grow in

sterol-free medium, the mollicute was transferred to a new genus, *Anaeroplasma*, that was created to accommodate strictly anaerobic mollicutes (Robinson and Allison, 1975; I. M. Robinson et al., 1975).

Mycoplasmatales

Facultatively anaerobic, sterol-requiring mollicutes are classified as *Mycoplasmatales*, the first order to be established among the mollicutes. This order contains two families, the *Mycoplasmataceae* and the *Spiroplasmataceae*. In those *Mycoplasma* and *Spiroplasma* spp. that have been examined, NADH oxidase activity is usually localized in the cytoplasm, whereas the activity is membrane-associated in the *Acholeplasmatales* (Pollack, 1975, 1978; Whitcomb and Tully, 1984). More recently, however, exceptions to this proclivity have been reported (Pollack et al., 1984*b*).

The *Mycoplasmataceae* The organisms in this family have genomes of 5 x 10^9 d in size (Razin and Freundt, 1984); this is the smallest known genome of any free-living organism (Stanbridge and Reff, 1979). The validity of these genome-size determinations has recently been questioned. Using pulse-field electrophoresis, Finch and co-workers (Pyle et al., 1988) determined that the genome sizes of *U. urealyticum* and *M. mycoides* subsp. *mycoides* were 0.6 x 10^9 d and 0.9 x 10^9 d, respectively. However, Maniloff (1989) maintains that DNA with low G+C content, like that of mollicute genomes, exhibits anomalous behavior when subjected to pulse-field electrophoresis.

The *Mycoplasmataceae* usually do not synthesize lipids from acetate (Herring and Pollack, 1974; Pollack et al., 1984*a*; Razin 1975, 1978). Romano, however, reported that some freshly isolated ureaplasmas have this ability but lose it upon subculturing (cited by Pollack et al., 1984*a*). Members of this family are usually parasites or pathogens of warm-blooded animals (Razin and Freundt, 1984).

The genus *Mycoplasma* The G+C contents of the DNA of *Mycoplasma* spp. range from 23 to 40 mol%. The G+C contents of most species are under 33 mol%; many are below 30 mol% (Freundt and Razin, 1984). Such a low G+C content of a genome is remarkable because the theoretical minimum necessary to code for proteins of normal amino acid composition is 26 mol% (Elton, 1973). *Mycoplasma* spp. may or may not ferment glucose and hydrolyze arginine; no species hydrolyzes urea (Freundt and Razin, 1984).

Because of their wall-less state, the membranes of mollicutes have been intensively studied both for the description of mollicutes and for the understanding of membrane biology in general (McElhaney, 1984, 1986; Razin, 1975, 1978). Mycoplasmas can be distinguished from the acholeplasmas by their ability to incorporate exogenous phospholipids (Razin et al., 1980; Rodwell, 1983; Rottem et al., 1986). Whereas most *Mycoplasma* spp. do not modify exogenous phospholipids, the biologically distinct, flask-shaped mycoplasmas (Kirchhoff et al., 1984) modify phosphatidylcholine to the disaturated state (Rottem et al., 1986). Mycoplasmas tend to have an unsaturated fatty acid residue at the number

one position of phosphatidylglycerol and a saturated moiety at the number two position; this is the converse of most organisms (Rottem and Markowitz, 1979). Razin (1982) suggested that the sterol requirement of mycoplasmas stems from the inability of most species to modulate membrane fluidity by *de novo* fatty acid synthesis or selective incorporation of appropriate fatty acids.

Many *Mycoplasma* spp. have been isolated from humans and animals with various pathological conditions. However, a definite pathogenic etiology has been established for fewer mycoplasmas. Some of the well-characterized mycoplasmal diseases of animals include: contagious bovine pleuropneumonia (*M. mycoides* subsp. *mycoides*); pneumonia, mastitis, and arthritis in cattle (*M. bovis*); contagious caprine pleuropneumonia (*M. mycoides*); contagious agalactia in sheep and goats (*M. agalactiae*); caprine infectious keratoconjunctivitis (*M. conjunctivae*); enzootic pneumonia of pigs (*M. hyopneumoniae*); nonsuppurative arthritis in pigs (*M. hyosynoviae*); respiratory infections in chickens and turkeys (*M. gallisepticum* and *M. synoviae*); and respiratory infections in rodents (*M. pulmonis*) (Jordan, 1979; Razin and Barile, 1985; Tully and Whitcomb, 1979).

In humans, the involvement of *M. pneumoniae* in primary atypical pneumonia has been unambiguously demonstrated (Razin and Barile, 1985; Tully and Whitcomb, 1979). There is good evidence to support the association of mycoplasmas with arthritis in immunosuppressed individuals and arthritic sequelae of *M. pneumoniae* infections (Razin and Barile,

1985). The role of *M. hominis* and *M. genitalium* in diseases of the urogenital tract is less clear (Razin and Barile, 1985).

Many more *Mycoplasma* spp. have been isolated from diseased animals, but their involvement as primary pathogenic agents is unclear. However, it should be noted that mycoplasmas and mollicutes in general may be important co-factors in pathological conditions caused by consortia of two or more microorganisms (Jordan, 1979; Lemcke, 1979).

The genus *Ureaplasma* These sterol-requiring mollicutes, with a genome size of 0.5 x 10^9 d, are distinguished from mycoplasmas by their very small colony size and by their unique ability among the mollicutes to hydrolyze urea (Shepard and Masover, 1979; Taylor-Robinson and Gourlay, 1984). The hydrolysis of urea to CO_2 and NH_3 by these organisms was reported simultaneously by Shepard and by Purcell et al. (cited by Shepard and Masover, 1979).

Research on ureaplasmas has been hampered by the yields of cells; maximum yields are 10^7 cells per ml (Razin, 1978). Yields were even smaller until it was determined that ureaplasmas have a lower pH optimum (pH 6.0) than that of mycoplasmas (pH 7.6 to 8.0) (Shepard and Masover, 1979). Ureaplasmas grow best under 5-100% CO_2, depending on the medium.

Ureaplasmas isolated from humans have DNA with G+C contents of 26.9 to 28.0 mol%, whereas the G+C contents of DNA of animal ureaplasmas range from 28.7 to 30.2 mol% (Taylor-Robinson and Gourlay, 1984). Human ureaplasmas, which cause nongonococcal urethritis, have been named *Ureaplasma urealyticum* (Shepard and Masover, 1979). Bovine strains are

classified as *Ureaplasma diversum* (Howard and Gourlay, 1982), while other animal ureaplasmas remain unclassified.

The *Spiroplasmataceae* (genus *Spiroplasma*) The other family in the order *Mycoplasmatales* is the *Spiroplasmataceae*, with its single genus *Spiroplasma*. These sterol-requiring organisms are distinguished by their helical morphology, rotatory motility, and their exclusive association with arthropods and plants (Whitcomb and Tully, 1984). The helical morphology is not apparent under all conditions. The nature of the helical shape and the motility in the absence of a rigid cell wall and flagella is an enigma that has not been resolved (Saglio and Whitcomb, 1979).

Unlike the other members of the *Mycoplasmatales*, spiroplasmas have a genome size of 1×10^9 d. In addition, spiroplasmas have an abundance of plasmids and episomes, unlike the other mollicutes (Nur et al., 1986; Ranhand et al., 1987; Razin, 1985). Like *Mycoplasma* spp. and some *Ureaplasma* spp., spiroplasmas cannot synthesize fatty acids from acetate (Whitcomb and Tully, 1984).

Spiroplasmas are associated with pathological conditions in a wide variety of hosts (Whitcomb and Tully, 1984): citrus stubborn disease (*S. citri*); corn stunt disease (*S. kunkelii*); May disease of honeybees (*S. apis*); lethargy disease of the *Melolontha* beetle (*S. floricola*); and maternally inherited male lethality in *Drosophila* (the spiroplasmal sex-ratio organism).

S. mirum has been associated with several human and animal pathological conditions, but no definite causal relationship has been established. *S. mirum* can experimentally cause cataracts in suckling mammals (Whitcomb and Tully, 1984), and this spiroplasma also has been reported to transform mouse and monkey cells in culture (Kotani et al., 1986). In addition, Bastian et al. (1987) reported that antisera to scrapie-associated fibril protein reacted with fibril protein from *S. mirum*, prompting these researchers to suggest a relationship between spongiform encephalopathy and spiroplasmal infection.

Acholeplasmatales (family ***Acholeplasmataceae***, genus ***Acholeplasma***)

Acholeplasmas are facultatively anaerobic, sterol-nonrequiring mollicutes that have membrane-associated NADH oxidase and a genome size of 1×10^9 d (Freundt et al., 1984; Tully, 1984). These organisms metabolize glucose, but they cannot hydrolyze arginine or urea. Unlike mycoplasmas, *Acholeplasma* spp. have lactate dehydrogenase that is activated by fructose-1,6-bisphosphate (Neimark and Tung, 1973). Acholeplasmas isolated from animals synthesize lipids from acetate, but insect- and plant-associated acholeplasmas cannot (Pollack et al., 1984*a*).

Acholeplasmas are able to modulate membrane fluidity by carotenoid synthesis, selective incorporation and elongation of fatty acids, and, in some instances, *de novo* synthesis of fatty acids. These abilities may be the basis of the sterol independence of the acholeplasmas (Razin, 1982).

Acholeplasmas are found in association with animals, plants, and insects (Tully et al., 1988). Although they are usually not considered pathogens, there are some indications of isolated cases of disease. For example, *A. axanthum* has been associated with disease in adult geese and ducks, as well as reduced hatchability of goose eggs (Tully and Whitcomb, 1979).

Anaeroplasmatales (family *Anaeroplasmataceae*)

When I. M. Robinson and Allison (1975) determined that the strictly anaerobic mollicute *Acholeplasma bactoclasticum* required sterol and thus placed it in a new genus *Anaeroplasma,* they also described the isolation from the rumen of other strictly anaerobic mollicutes. These strictly anaerobic mollicutes were nonbacteriolytic, and those that required sterol were placed in the new species *Anaeroplasma abactoclasticum* (Robinson et al., 1975). Other nonbacteriolytic strains were *bona fide* sterol-nonrequiring organisms. Robinson et al. (1975) did not name these sterol-nonrequiring strains, nor did they assign the new genus *Anaeroplasma* to a higher taxon, because they believed that the strictly anaerobic nature and other similiarities of these organisms did not substantiate the division of this group based on sterol requirement.

Because sterol-requirement had always been an important criterion in the classification of the mollicutes, the suggestion that the sterol-requiring and -nonrequiring strains of the strictly anaerobic mollicutes be classified together met with opposition (Subcommittee, 1977).

However, further work determined that the strictly anaerobic mollicutes were serologically distinct from bovine mycoplasmas and acholeplasmas (Robinson and Rhoades, 1977). The various sterol-requiring strains of the two species of *Anaeroplasma* could be divided into four distinct species by DNA-DNA hybridization experiments; there was little homology with DNA from *M. hominis* (Stephens et al., 1985). Similarly, the DNA from the sterol-nonrequiring, strictly anaerobic mollicutes did not hybridize significantly to the DNA from nine *Acholeplasma* spp. (Stephens et al., 1985).

Although the sterol-requiring and -nonrequiring, strictly anaerobic mollicutes could not be classified in the same genus, the serological and DNA hybridzation experiments demonstrated that these organisms were distinct from the *Mycoplasmatales* and the *Acholeplasmatales*. Furthermore, while the sterol-requiring *Mycoplasmatales* have a genome size of 0.5 x 10^9 d, and the sterol-nonrequiring *Acholeplasmatales* have a genome size of 1 x 10^9 d, both the sterol-requiring and -nonrequiring, strictly anaerobic mollicutes had a genome size of 1 x 10^9 d (Christiansen et al., 1986).

Based on these investigations, Robinson and Freundt (1987) made three taxonomic proposals. First, in addition to *An. bactoclasticum* and *An. abactoclasticum*, two new species (*An. intermedium* and *An. varium*) were created to accommodate the clustering of the sterol-requiring strains as determined by DNA homology (Stephens et al., 1985). Second, a new genus with a single species, *Asteroleplasma anaerobium*, was created

for sterol-nonrequiring, strictly anaerobic mollicutes. Third, the genera *Anaeroplasma* and *Asteroleplasma* were placed in a new family (the *Anaeroplasmataceae*) and a new order (the *Anaeroplasmatales*). Thus, all the strictly anaerobic mollicutes are classified together, but they remain divided at the genus level based on sterol requirement.

The anaeroplasmas and asteroleplasmas have a G+C content of 29.3 to 33.7 mol% and 40.2 to 40.3 mol%, respectively (Robinson and Freundt, 1987). The strictly anaerobic mollicutes ferment carbohydrates but do not hydrolyze arginine or urea, and they do not synthesize lipid from acetate (Robinson, 1973, 1979, 1984; Robinson and Freundt, 1987; Robinson et al., 1975). Like other strictly anaerobic bacteria, *Anaeroplasma* spp. contain plasmalogens (Langworthy et al., 1975). The proteolytic and bacteriolytic abilities of an anaeroplasma were studied by J. P. Robinson (1971), and he tentatively concluded that a single enzyme was responsible for both activities. Thusfar, all anaerobic mollicutes have been isolated from cattle and sheep; attempts to isolate them from wild ruminants (deer) and monogastric animals have been unsuccessful (J. P. Robinson, 1971; I. M. Robinson et al., 1975). Rose and Pirt (1981) reported the isolation of an anaeroplasma from sewage sludge, but some mycoplasmologists have expressed concern about the validity of this organism as a mollicute, as well as a second isolate of Rose and Pirt, "*Methanoplasma elizabethii*" (Board of Directors, 1983; Subcommittee, 1984).

Mycoplasma-like organisms

Organisms that have typical mollicute morphology have been observed by electron microscopy in sieve-tube elements of plants with yellows diseases and in the salivary glands of associated insect vectors (McCoy, 1984). These mycoplasma-like organisms (MLO) have not been cultured, and thus, little is known about their biology beyond the symptomology of the associated diseases and their transmission by insects (McCoy, 1984; Whitcomb and Tully, 1979). However, the recent cultivation of the spiroplasma responsible for the sex-ratio trait in *Drosophila,* 25 years after its discovery (Hackett et al., 1986), indicates that further progress on the study of MLO is possible.

Further reading

Many aspects of the mollicutes are discussed in detail in *Bergey's Manual of Systematic Bacteriology* (Razin and Freundt, 1984) and in the four-volume set, *The Mycoplasmas: I. Cell biology* (Barile and Razin, 1979); *II. Human and animal mycoplasmas* (Tully and Whitcomb, 1979); *III. Plant and insect mycoplasmas* (Whitcomb and Tully, 1979); and *IV. Mycoplasma pathogenicity* (Razin and Barile, 1985). Methodology is discussed in the two-volume set, *Methods in Mycoplasmology: I. Mycoplasma characterization* (Razin and Tully, 1983) and *II. Diagnostic mycoplasmology* (Tully and Razin, 1983). Readers interested in specific areas of mycoplasmology may consult Hayflick (1969) and Smith (1971) (history); Barile et al. (1985) and Bové and Tully (1984) (pathogenicity

and control of mollicutes); and Razin (1985) (molecular biology).

Physiology of the *Mollicutes*

The physiology and genetics of mollicutes are of considerable interest because these organisms are the smallest, free-living biological entities known. Morowitz (1984) proposed that mollicutes should be thoroughly studied on the physiological and genetic levels to determine the minimal requirements for independent life, or, as Morowitz refers to it, "the logic of life".

Two major facets of physiology are energy production and biosynthesis. The literature dealing with lipid metabolism in mollicutes (binding and incorporation, modification, *de novo* synthesis, membrane proteins and fluidity, and transport) is voluminous. I did not examine lipid metabolism, and thus, it will not be discussed further. Readers interested in this area may consult McElhaney (1984, 1986) and Razin (1975, 1978).

The bulk of the metabolic studies of mollicutes has been conducted with *Mycoplasma* and *Acholeplasma* spp. Less is known about ureaplasmas, spiroplasmas, and especially the strictly anaerobic mollicutes. J. P. Robinson and R. E. Hungate (1973) determined that *Acholeplasma bactoclasticum* ATCC 27112 [*Anaeroplasma bactoclasticum* (Robinson and Allison, 1975)] fermented a variety of carbohydrates; acetate, formate, lactate, CO_2, and hydrogen were produced from galactose. I. M. Robinson and colleagues (1973, 1975, 1979, 1984) reported that the strictly

anaerobic mollicutes fermented starch, producing acetate, formate, lactate, ethanol, and CO_2. Production of hydrogen, succinate, and propionate, and stimulation of growth by different monosaccharides and disaccharides varied between strains.

Except for the study of acetate kinase activity in mollicutes by Muhlrad et al. (1981), the intracellular metabolism of carbohydrates by the strictly anaerobic mollicutes has not been examined. Muhlrad et al. (1981) reported that *Anaeroplasma abactoclasticum* 6-1^T and *Anaeroplasma intermedium* 7LA^T did not have acetate kinase activity. Thus, the mechanism by which these strains produce acetate (Robinson and Allison, 1975; Robinson et al., 1975) is unclear.

McElwain et al. (1988) examined other intracellular enzymic activities of eight mollicutes including *An. intermedium* 5LA. These findings will be discussed in the section on purine and pyrimidine metabolism of mollicutes.

The only other studies of the physiology of strictly anaerobic mollicutes were the characterization of the extracellular bacteriolytic and proteolytic activities of *An. bactoclasticum* and the extracellular amylase of *An. bactoclasticum* 5LA [*An. intermedium* (Robinson and Freundt, 1987)]. Some of the anaerobic mollicutes can lyse Gram-negative cells that have been previously disrupted by heat, freeze-drying, or other means (J. P. Robinson, 1971; J. P. Robinson and Hungate, 1973). Robinson (1971) also noted that *Acholeplasma* (*An.*) *bactoclasticum* ATCC 27112 was proteolytic. Both activities were reversibly sensitive to O_2, and

Robinson tentatively concluded that the proteolytic and bacteriolytic activities were the same enzyme.

Jost (1979) developed a medium for the production of an extracellular amylase by *An. bactoclasticum* (*intermedium*) 5LA and partially purified the enzyme. The most salient characteristics of the enzyme were its high degree of activity on maltotriose compared with other amylases and the fact that the main products were glucose and maltose.

In contrast to the paucity of information about the metabolism of the strictly anaerobic mollicutes, the *Mycoplasmatales* and the *Acholeplasmatales* are better-characterized. With the exception of *Acholeplasma parvum* (Atobe et al., 1983), all acholeplasmas and spiroplasmas, and many mycoplasmas, ferment glucose (Razin and Freundt, 1984). Fermentative mycoplasmas internalize glucose via a PEP-dependent phosphotransferse system, whereas acholeplasmas and nonfermentative mycoplasmas do not have this activity (Rottem and Cirillo, 1986). Rodwell and Rodwell (1954*a*) demonstrated that suspensions of *M. mycoides* subsp. *mycoides* aerobically catabolized glucose, pyruvate, and lactate to acetate and CO_2; under anaerobic conditions, pyruvate was catabolized to lactate, acetate, and CO_2. Subsequently, other workers reported that glucose and other carbohydrates were catabolized to lactate, acetate, CO_2, and other products in a variety of mycoplasmas (Neimark and Pickett, 1960; Tourtellotte and Jacobs, 1960). Rodwell and Rodwell (1954*b*) also detected aldolase in *M. mycoides* subsp. *mycoides*, and they assumed that

the Embden-Meyerhof-Parnas (EMP) pathway was responsible for the degradation of glucose. Castrejon-Diez et al. (1963) detected activities for hexokinase, aldolase, and glyceraldehyde 3-*P* dehydrogenase in *A. laidlawii* and concluded that the EMP pathway was active in this organism. However, the recovery of $^{14}CO_2$ during the metabolism of [1-^{14}C]glucose and the demonstration of glucose-6-*P* (Glc-6-*P*) dehydrogenase, ribulose-5-*P* isomerase, and transketolase activities indicated that the pentose phosphate pathway was also active in *A. laidlawii* (Castrejon-Diez et al., 1963).

Some enzymes associated with the EMP pathway have been detected in a variety of mollicutes by electrophoretic fractionation of intracellular contents and specific staining methods. These enzymes include Glc-6-*P* isomerase, aldolase, triose phosphate isomerase, phosphoglycerate kinase, enolase, and pyruvate kinase (Lanham et al., 1980; O'Brien et al., 1981; Salih et al., 1983). *Acholeplasma* spp. also had activities for Glc-6-*P* dehydrogenase and 6-phosphogluconate (6-PG) dehydrogenase, enzymes associated with the shunt portion of the pentose phosphate pathway (Lanham et al., 1980; Salih et al., 1983).

These studies that used electrophoretic fractionation of isozymes for taxonomic and epidemiological purposes gave important indications of the metabolic processes involved, but information about the organismal biology was still piecemeal. In the past several years, cell-free extracts of mollicutes have been examined more extensively to determine the presence or absence of metabolic pathways. Such investigations are

more appropriate for the determination of metabolic maps necessary for the proposed investigation of the coding capacity of the mollicute genome (Morowitz, 1984; Razin, 1985).

The first organisms investigated in this manner were *U. urealyticum* and *M. mycoides* subsp. *mycoides* (Cocks et al., 1985). *M. mycoides* subsp. *mycoides* had activities for ten enzymes of the EMP pathway, leading from glucose to pyruvate and then to lactate and acetate. ATP-dependent phosphofructokinase (ATP-PFK) was one of these enzymes detected; this is the first report of this enzyme in a mollicute. Because PFK is unique to the EMP pathway, it is a specific indicator of this pathway (Gottschalk, 1986). Cocks et al. (1985) did not detect all enzymes of the EMP pathway in *U. urealyticum*. Activities for ATP-PFK, aldolase, triose phosphate isomerase, glyceraldehyde-3-*P* dehydrogenase, enolase, and pyruvate kinase were present, but hexokinase, Glc-6-*P* isomerase, phosphoglycerate kinase, and phosphoglycerate mutase were not detected. Because *U. urealyticum* does not ferment glucose, Cocks et al. (1985) suggested that the limited EMP pathway enzymes may be functional for the formation of ribose 5-*P* from glycolytic intermediates for nucleoside synthesis rather than as a major energy-yielding pathway. Substantiating evidence for this function is the presence of the nonoxidative portion of the pentose phosphate pathway in this organism (Cocks et al., 1985). *M. mycoides* subsp. *mycoides* also had activities for enzymes of the nonoxidative portion of the pentose phosphate pathway (Cocks et al., 1985). Pollack (1986) examined *U. urealyticum* for glycolytic enzymes; hexokinase, Glc-6-*P*

isomerase, and aldolase were not detected, and only trace amounts of ATP-PFK were observed.

DeSantis et al. (1989) examined 18 fermentative and nonfermentative *Mycoplasma* and *Acholeplasma* strains for enzymes of the EMP and pentose phosphate pathways. The prominent distinctions among the mycoplasmas were: (i) the absence in the nonfermentative species of all pentose phosphate pathway enzymes except ribulose-5-*P* epimerase and, (ii) the presence in the nonfermentative species of only the last portion of the EMP pathway, metabolizing three-carbon compounds [glyceraldehyde 3-*P* to phospho*enol*pyruvate (PEP)]. In contrast, fermentative mycoplasmas had activities for the nonoxidative portion of the pentose phosphate pathway, and these species had an almost-complete EMP pathway, including ATP-PFK and aldolase; hexokinase was not detected.

Two observations by DeSantis et al. (1989) distinguished the acholeplasmas from the mycoplasmas. First, only the *Acholeplasma* spp. had activities for the oxidative enzymes of the pentose phosphate pathway (Glc-6-*P* dehydrogenase and 6-PG dehydrogenase), confirming the observations of O'Brien et al. (1981). Both of these reactions generate NADPH, an important cofactor in lipid biosynthesis. The absence of these reactions in *Mycoplasma* spp. may relate to their greater need for exogenous lipids (DeSantis et al., 1989). The second distinctive feature of the acholeplasmas was the enzyme PFK, which was active with pyrophosphate (PP_i) but not with ATP (DeSantis et al., 1989; Pollack and Williams, 1986). The majority of bacterial PFKs are active with ATP;

PP_i-dependent PFK has been reported only in *Propionibacterium freundenreichii* and *P. acidipropionici* ("*P. shermanii*") (O'Brien et al., 1975; Wood and Goss, 1985), some *Bacteroides* spp. (Macy et al., 1978; Roberton and Glucina, 1982), *Deleya* spp. (Sawyer et al., 1977), and a *Clostridium* sp. isolated from the cockroach gut (Cruden et al., 1983).

DeSantis et al. (1989) detected hexokinase in the four *Acholeplasma* spp., but this enzyme was absent in 13 of 14 *Mycoplasma* strains. This is in agreement with the reported occurrence of the PEP-dependent phosphotransferase system among mycoplasmas but not acholeplasmas (Rottem and Cirillo, 1986). However, Rodwell and Rodwell (1954*b*) reported evidence suggestive of hexokinase activity in *M. mycoides* subsp. *mycoides;* this was confirmed by Cocks et al. (1985). Because of its pathogenicity, *M. mycoides* subsp. *mycoides* is not available in the United States and thus was not studied by DeSantis et al. (1989). In addition, *Mycoplasma* sp. Lett 1, isolated from lettuce, had hexokinase activity; this isolate is also distinct from other *Mycoplasma* spp. in several other characteristics (Pollack et al., 1984*b*).

Spiroplasmas, which ferment glucose, have activities for the EMP pathway, including ATP-PFK (Lake-Bullock et al., 1989; Pollack et al., 1989). Like the other *Mycoplasmatales,* spiroplasmas lack the oxidative enzymes of the pentose phosphate pathway, Glc-6-*P* dehydrogenase and 6-PG dehydrogenase (Lake-Bullock et al., 1989; Pollack et al., 1989). Pollack et al. (1989) detected all the other enzymes of the pentose phosphate pathway in *S. apis, S. citri, S. culicicola, S. melliferum,* and

S. sabaudiense; S. kunkelii and *S. murium* lacked transaldolase activity. Lake-Bullock et al. (1989) examined *S. apis, S. citri, S. floricola,* and *S. melliferum;* they detected transaldolase and transketolase in all these species. Thus, the spiroplasmas share the distinctions that set the other *Mycoplasmatales* apart from the *Acholeplasmatales.*

Egan et al. (1986) used ^{31}P-NMR to examine the catabolism of glucose in intact cells of *M. gallisepticum.* The glycolytic intermediates Glc-6-*P*, fructose-1,6,-P_2, and PEP were detected, but other phosphorylated intermediates were not.

Enzymes of the tricarboxylic acid (TCA) cycle and enzymes leading to the cycle have been examined in a number of mollicutes. The TCA cycle is potentially important for energy production and for providing carbon skeletons for biosynthesis. VanDemark and Smith (1964*b*) reported that a single strain of *M. hominis* (now classified as *M. arthriditis*) possessed five TCA cycle enzymes; citrate synthase was not detected. However, Lecce and Morton (1954), Rodwell and Rodwell (1954*a*), and Tourtellette and Jacobs (1960) reported that mollicutes do not actively oxidize TCA cycle intermediates.

Mollicutes have been examined for some TCA cycle enzymes by several groups that used analysis of isozymes in taxonomic and epidemiological studies. O'Brien et al. (1981) did not detect isocitrate dehydrogenase or malate dehydrogenase in 22 *Mycoplasma* and *Acholeplasma* spp. examined by electrophoretic fractionation of cell extracts followed by staining. Using similar techniques, Lanham et al. (1980) did not detect either of

these two enzymes in four stains of *A. laidlawii*. Salih et al. (1983), who also used electrophoretic isozyme analysis, reported the presence of weak activity for malate dehydrogenase in *Acholeplasma* spp., and Delisle (1977) reported the presence of this enzyme in *U. urealyticum*.

Manolukas et al. (1988) conducted a more extensive examination of cell-free extracts of ten mycoplasmas and acholeplasmas for enzymes of the TCA cycle. No TCA-cycle enzymes except malate dehydrogenase were detected in *Mycoplasma* spp., and the two *Acholeplasma* spp. lacked all the TCA cycle enzymes. The two acholeplasmas could carboxylate PEP to oxaloacetate, but the mycoplasmas could not (Manolukas et al., 1988). Constantopoulos and McGarrity (1987) detected pyruvate dehydrogenase activities in *A. laidlawii* and four *Mycoplasma* spp., but these species had little or no α-ketoglutarate dehydrogenase activity. Davis et al. (1988, 1989) did not detect any TCA cycle enzymes except malate dehydrogenase in ureaplasmas; Pollack et al. (1989) reported similar observations with *Spiroplasma* spp. Thus, all three genera in the family *Mycoplasmataceae* have malate dehydrogenase activity.

These multiple reports of the absence of an intact TCA cycle in a variety of mollicutes contrast with VanDemark and Smith's report (1964*b*) of the presence of the TCA cycle in a single strain of *M. arthriditis*. VanDemark and Smith (1964*a*) also reported the presence of cytochromes and quinones and oxidative phosphorylation in this strain. However, Holländer et al. (1977) could not confirm the presence of cytochromes in this strain, and they could only detect minute amounts of quinones. This

report, the absence of detectable cytochromes in 14 mollicutes reported by Pollack et al. (1981), and the multiple reports cited by Pollack (1979) of unsuccessful attempts to find cytochromes in several mollicutes, diminish the importance of VanDemark and Smith's (1964*a*,*b*) findings as they apply to mollicutes in general (Razin, 1978).

Because most mollicutes lack the TCA cycle, they may be limited in their ability to synthesize amino acids derived from TCA-cycle intermediates. However, some mollicutes may be able to synthesize the aspartate family of amino acids. Many mollicutes possess pyruvate carboxylase and aspartate aminotransferase activities (Davis et al., 1988, 1989; Manolukas et al., 1988), and thus, they may be able to convert pyruvate to oxaloacetate and then to aspartate. In addition, Tourtellotte (cited by Rodwell and Mitchell, 1979) determined that *A. laidlawii* may possess the shikimate pathway for aromatic amino acid biosynthesis because this organism could incorporate ^{14}C-labelled shikimate into protein. Berry et al. (1987) examined this pathway in more detail. *A. laidlawii* possessed DHAP synthase, shikimate dehydrogenase, 3-phosphoshikimate 1-carboxyvinyltransferase, prephenate dehydratase, prephenate dehydrogenase, and chorismate mutase. *M. iowae* and *M. gallinarum* are more typical of most mollicutes in that they do not possess any enzymic activities associated with this pathway (Berry et al., 1987).

In fact, though, mollicutes require the inclusion of multiple amino acids in partly or completely defined media (Patterson et al., 1985;

Rodwell, 1969; Rodwell and Mitchell, 1979; Smith, 1971). Some amino acids are more readily assimilated by mollicutes if they are supplied as short peptides (Rodwell, 1969). In this form, potential antagonistic competitions for common transport systems by free amino acids are avoided.

Mollicutes produce aminopeptidases and carboxypeptidases (Ball et al., 1982; Choules and Gray, 1971; Shibata and Watanabe, 1986). These may supply the organisms with amino acids for protein synthesis or, in the case of arginine, energy (Shibata and Watanabe, 1986).

The absence of the TCA cycle in most mollicutes has a second physiological consequence. The mollicutes have limited abilities to generate reduced pyridine coenzymes for energy production. However, Pollack (1979) summarized the oxidative reactions that generate reducing equivalents that have been reported to occur among the mollicutes. These include Glc-6-*P* dehydrogenase, α-glycerophosphate dehydrogenase, glyceraldehyde-3-*P* dehydrogenase (NADH and NADPH), lactate dehydrogenase, malate dehydrogenase, glutamate dehydrogenase (NADH and NADPH), alcohol dehydrogenase, hydroxymethyl glutaryl-CoA reductase (NADPH), and β-hydroxybutyrate dehydrogenase. These enzymic activities have either been detected directly, or the oxidation of the respective substrates by mollicutes has been reported (Pollack, 1979).

Because mollicutes in general lack cytochromes, the reduced pyridine nucleotides can only be used for the production of energy by donating their electrons to a flavin-terminated respiration chain. In contrast to

the absence of cytochromes, flavins or flavoproteins occur more widely among mollicutes (Pollack, 1979; Pollack et al., 1981). The first report of flavin-associated oxidations was by Smith et al. (1963). Morowitz and Terry (1969) detected characteristic spectra of flavins in *A. laidlawii*. Reinards et al. (1981) purified a flavoprotein with NADH oxidase activity from *A. laidlawii*; the enzyme transferred electrons from NADH to O_2, ferricyanide, and dichloroindophenol. Gaisser et al. (1987) determined that copper ions inhibit lactate dehydrogenase and NADH oxidase, thereby inhibiting the growth of *M. gallisepticum* by interfering with these energy-yielding reactions.

With respiratory-linked production of ATP limited to flavin-associated reactions, the importance of substrate-level phosphorylation in mollicutes increases (Pollack et al., 1983). Pyruvate kinase, as part of the end of the EMP pathway, converts PEP to pyruvate, concommitantly generating ATP. This enzyme has been reported in a variety of fermentative mycoplasmas, ureaplasmas, spiroplasmas, and acholeplasmas (Cocks et al., 1985; Davis et al., 1988, 1989; Lake-Bullock et al., 1989; Lanham et al., 1980; Manolukas et al., 1988; Pollack et al., 1989). Another ATP-generating enzyme, acetate kinase, has also been detected in a number of mollicutes (Castrejon-Diez et al., 1962; Kahane et al., 1978; Muhlrad et al., 1981; Rottem and Razin, 1967; Smith and Henrikson, 1965).

A few other substrate-linked phosphorylation activities have been reported in a more limited number of mollicutes. The arginine dihydrolase pathway converts arginine to citrulline, which then undergoes

phosphorolysis to yield ornithine and carbamoyl phosphate. In turn, ATP is generated from carbamoyl phosphate. Among the mollicutes, this pathway was first reported by Smith (1957). Since then, it has been found in both fermentative and nonfermentative *Mycoplasma* spp. (Freundt and Razin, 1984) and spiroplasmas (Igwegbe and Thomas, 1978; Whitcomb and Tully, 1984). Arginine hydrolysis has not been reported in ureaplasmas, acholeplasmas, or the strictly anaerobic mollicutes (Razin and Freundt, 1984).

In those mollicutes that hydrolyze arginine, this substrate was at one time thought to be the major energy-yielding pathway, but subsequent findings diminish this possibility. Hahn and Kenny (1974) determined that some arginine-hydrolyzing mycoplasmas do not require arginine to initiate growth. Furthermore, Fenske and Kenny (1976) reported that arginine deiminase is not present at significant levels until the late-logarithmic stage of growth of *M. hominis*, and thus, arginine hydrolysis cannot be the primary source of energy.

Because ureaplasmas neither ferment glucose nor hydrolyze arginine, it has been speculated that they derive energy from the hydrolysis of urea. The carbon and nitrogen from the breakdown of urea are not incorporated, but Masover et al. (1977) suggested that urea hydrolysis could generate ATP indirectly by a chemiosmotic mechanism. According to this model, ammonium ions derived from urea diffuse out of the cell, creating a proton gradient. Protons then enter the cell, leading to ATP generation by a membrane-bound ATPase. Romano et al. (1980) provided

experimental evidence supportive of this hypothesis by demonstrating that the addition of urea to resting ureaplasma cells resulted in its hydrolysis and a concomitant rise in levels of intracellular ATP. This activity was dependent on urease and membrane-bound ATPase; the dissipation of the proton gradient by carbonyl cyanide-*m*-chlorophenylhydrazone inhibited the generation of ATP. In addition, high external pH diminshed the generation of ATP (Romano et al., 1986).

Various workers have demonstrated that mollicutes regulate ion concentration and cell volume by the creation of membrane potentials; this requires the expenditure of ATP (Clementz et al., 1987; Rottem et al., 1981; Schummer and Schiefer, 1987). Such osmotic controls are especially important in a wall-less organism.

The limited number of mechanisms to generate ATP and the concomitant accentuated energy demands imposed by the wall-less condition may be responsible for the low intracellular adenylate energy charges (E_c) observed in several mollicutes. *S. citri* (Saglio et al., 1979) and *A. laidlawii* (Beaman and Pollack, 1981) had E_c values similar to walled bacteria (0.90), but *A. morum* and four *Mycoplasma* spp. had E_c values of approximately 0.70 (Beaman and Pollack, 1983). Clementz et al. (1987) reported that *A. laidlawii* also had low E_c values under certain conditions that altered membrane lipid composition. Beaman and Pollack (1983) suggested that because mollicutes have a relative energy deficit, they may be energy parasites *in vivo*, similar to rickettsiae and chlamydiae. This hypothesis is supported by the observation of Gabridge

and Stahl (1978) that the addition of adenine, adenosine, or ATP decreased the cytopathic effects of *M. pneumoniae* on tissue cultures.

Mollicutes have also been examined for nucleic acid metabolism. Most culture media for mollicutes are supplemented with DNA, and many mollicutes possess one or more nucleases that can degrade DNA to a more assimilable form (Pollack and Hoffmann, 1982). *M. mycoides* subsp. *mycoides* has the unusual ability to directly incorporate deoxyribomononucleotides and subsequently phosphorylate them to the triphosphate form (Neale et al., 1984).

Some mollicutes, such as *M. mycoides* subsp. *mycoides* cannot synthesize ribonucleotides *de novo* (Mitchell and Finch, 1977); their nutritional requirements are met by nucleobases. Tryon and Pollack (1984) reported that *A. laidlawii* did not incorporate [U-^{14}C]glycine, and they therefore concluded that this organism could not synthesize purine bases *de novo*. Because most mollicutes possess the enzymes of the nonoxidative portion of the pentose phosphate pathway, they can form ribose-5-*P* from glucose, and thus, they can probably synthesize nucleotides from glucose and nucleobases (Cocks et al., 1985; DeSantis et al., 1989). The occurrence of dihydrofolate reductase in *M. gallisepticum* and *A. laidlawii*, an enzyme important in the synthesis of purines and thymidylate (Mandelbaum-Shavit and Kahane, 1988), indicates that some mollicutes have the anabolic potential for *de novo* synthesis of nucleobases. Thusfar, however, no mollicute has been demonstrated to be capable of *de novo* synthesis.

In contrast to the questionable ability of mollicutes for *de novo* synthesis of nucleobases, mollicutes have enzymic activities that make them more proficient at purine and pyrimidine salvage and interconversions (Cocks et al., 1988; Hamet et al., 1980; McElwain and Pollack, 1987; McElwain et al., 1988; Mitchell and Finch, 1977; Neale et al., 1983; Tryon and Pollack, 1984, 1985; Williams and Pollack, 1985). They are able to interconvert purine nucleobases, (deoxy)ribonucleosides, and (deoxy)ribonucleotides; they are also able to interconvert pyrimidine deoxyribomono-, deoxyribodi-, and deoxyribotrinucleotides.

In regard to the one strictly anaerobic mollicute studied, *An. intermedium* 5LA was similar to other mollicutes in its capacity to interconvert purines, and the nucleoside kinase in this organism was PP_i-dependent (McElwain et al., 1988). PP_i-dependent nucleoside kinase has only been detected in some mollicutes; the nucleoside kinase in all other organisms examined is ATP-dependent (McElwain et al., 1988; Tryon and Pollack, 1984). Like other mollicutes (Cocks et al., 1988; McElwain et al., 1988; Neale et al., 1983), *An. intermedium* has thymidine phosphorylase activity and thus is able to produce thymidine nucleotides by the salvage pathway (McElwain et al., 1988). *An. intermedium* also has dCMP deaminase activity (McElwain et al., 1988). Thus, this organism and other mollicutes like it can derive deoxyribose-1-*P* for the thymidine salvage pathway by converting dCMP to dUMP and then deribosylating dUMP (McElwain et al., 1988; Neale et al., 1983).

Overall, mollicutes are limited in their capacity to generate energy and precursors for amino acid, lipid, nucleic acid, and vitamin synthesis. Thus, they are nutritionally fastidious and relatively slow-growing. These diminished metabolic capabilities may be a reflection of their small genomes and the associated constraint on the amount of encoded genetic information.

Phylogeny of the *Mollicutes*

Because of their extremely small size and filterability, some of the first mollicutes isolated were thought to be similar to etiological agents that are now known to be viruses. However, mollicutes were eventually recognized as a phenotypically similar group of bacteria (though radically different from walled bacteria), and they were called PPLO (pleuropneumonia-like organisms). With the observations by Klieneberger (1935) and Dienes (1938) of the walled bacterium *Streptobacillus moniliformis* in the L-phase of growth (L-forms), the identities of PPLO and L-forms became associated and intertwined with one another, causing great confusion (Smith, 1971).

The observations of Pierce (1942), that L-forms could be derived from walled bacteria with penicillin, and of Sharp (1954), that hypertonic media were indispensable for the recovery of L-forms, led to the concept that the L-phase is a general property of most bacterial species. Mollicutes were thought by some to be stable L-forms derived from recognized species. However, the use of DNA-DNA hybridization

studies in the 1960s demonstrated that although the DNA of an L-form hybridized with the DNA of the walled bacterium from which it was derived, mollicutes had no DNA homology with any of the walled bacteria studied (McGee et al., 1967).

Because mollicutes had small cell and genome sizes and limited metabolic capabilities, these organisms were proposed by Wallace and Morowitz (1973) to be primitive organisms decended from a progenitor organism from which both prokaryotes and eukaryotes arose. This model would require substantial increases in genome size; this conflicts with other theories that bacterial genomes tend to be "streamlined" (Doolittle, 1978). It must also assume that the primitive progenitors were capable of saprophytic existence independent of animal hosts, unlike many of the mollicutes.

Alternatively, it has been suggested that the mollicutes represent a collection of unrelated organisms that have degenerated from different walled bacteria (Dienes and Weinberger, 1951). Neimark (1979; Neimark and London, 1982) described a modified version of this latter scenario of degenerative evolution. He noted that several similarities existed between the glycolytic enzymes of acholeplasmas and those of lactic acid bacteria. Unlike mycoplasmas, the lactate dehydrogenase (LDH) of acholeplasmas is activated by fructose-1,6-bisphosphate (Fru-1,6-P_2), an allosteric phenomenon found only in some *Lactobacillaceae* (Neimark, 1979; Neimark and Tung, 1973). [Since those reports, (Fru-1,6-P_2)-activated LDH has been detected in *Clostridium thermohydrosulfuricum* (Turunen et

al., 1987)]. Furthermore, antisera prepared to aldolase and glyceraldehyde-3-*P* dehydrogenase purified from lactic acid bacteria reacted with extracts of acholeplasmas but not fermentative mycoplasmas, indicating that a high degree of immunological similarity existed between the enzymes from acholeplasmas and lactic acid bacteria (Neimark and London, 1982). Based on these findings, Neimark concluded that the acholeplasmas evolved from the streptococci by loss of the cell wall and genome reduction; mycoplasmas were proposed to have evolved from a different (unspecified) group of walled bacteria (Neimark, 1979; Neimark and London, 1982).

Woese and colleagues (1980) also proposed that the mollicutes were not primitive progenitors to walled bacteria, but instead that they evolved from walled bacteria. They based their conclusions on the analysis of 16S-rRNA sequences of bacteria. As part of a larger effort to develop catalogs of 16S-rRNA sequences in an attempt to determine phylogenetic relationships among bacteria in general, Woese et al. (1980) concluded that mycoplasmas, spiroplasmas, and acholeplasmas arose by degenerative evolution from an ancient clostridial group that also gave rise to the present-day bacillus-lactobacillus-streptococcus group. The mollicutes were most closely associated with *Clostridium innocuum* and *C. ramosum*, with the acholeplasmas being more closely related than the other mollicutes to these walled bacteria (Woese et al., 1980). The finding that the acholeplasmas are more closely related to the two clostridia than to the streptococci is in disagreement with Neimark's proposal.

The phylogenetic relationship between some walled bacteria and a greater number of mollicutes was re-examined by Woese and colleagues using 5S-rRNA analyses (Rogers et al., 1985). Whereas the 5S-rRNA molecules from Gram-negative bacteria have 120 nucleotides and those from Gram-positive bacteria are 116 to 117 nucleotides in length, the 5S-rRNA molecules of the mollicutes and their walled relatives were 104 to 114 nucleotides in length. Analysis of the 5S-rRNA sequences by Rogers et al. (1985) indicated that all the mollicutes form a coherent phylogenetic branch of the Gram-positive bacteria; this conflicts with Neimark's assertion that the mycoplasmas and acholeplasmas have little or no phylogenetic relatedness (Neimark, 1979).

Rogers et al. (1985) also proposed a general outline of the descent of the mollicutes. Through chromosomal deletions, the ancestors of the acholeplasmas arose from the clostridial branch. This group then split into the acholeplasma and spiroplasma branches; the anaeroplasmas branched off very close to this split, and their exact placement is uncertain. Within the spiroplasma branch, there were further genome reductions, leading to the mycoplasmas and ureaplasmas.

Since the study of Rogers et al. (1985), rRNA catalogs of more bacteria have been determined, and four more of these walled species have been found to be specifically related to the mollicutes: *Erysipelothrix rhusiopathiae* and *Lactobacillus catenaformis* [*L. catenaforme*, (Woese et al., 1985)], *Streptococcus pleomorphus* (Ludwig et al., 1988), and *Lactobacillus vitulinus* (Weisburg et al., 1989). In addition,

Bacteroides termiditis and an unnamed strain 23 [termed "pseudobacteroides" by Woese et al. (1985)] may be distantly related to the mollicutes, but they also have certain features that distinguish them from Gram-positive eubacteria.

The rRNA analyses of Woese and colleagues have also revealed that although most eubacteria have regions of highly conserved sequences in their rRNA, many of these conserved sequences are absent in the rRNA of the mollicutes (Rogers et al., 1985; Woese, 1987; Woese et al., 1980, 1985). While this makes the determination of binary association coefficients of relatedness more difficult, it also has important evolutionary implications that may explain some of the distinctive features of the mollicutes. Because rRNA molecules are necessary for translation (one of the earliest and most crucial biological processes), rRNAs are highly refined molecules that show relatively little variation in secondary structure between kingdoms (Woese, 1987). Any single mutation would probably create a mismatch that would disrupt the secondary structure of the molecule. However, two mutations that occur concomitantly (or nearly so) in paired positions have the potential to be less deleterious for the form and function of the rRNA molecules (Woese, 1987). Such paired (composite) mutations are higher-order functions of the organism's mutation rate (Woese 1987; Woese et al., 1985). In most organisms, the mutation rate is not high enough to allow composite mutations. However, in organisms with higher mutation rates, typical rRNA variants arise more frequently, but more importantly, other types of

rRNA variants will occur; these atypical variants arise with inappreciable frequency in organisms with more typical mutation rates (Woese, 1987; Woese et al., 1985). Woese and his colleagues proposed that mollicutes have an elevated rate of mutation; this is responsible for the bizarre phenotypes of mollicutes and for their rRNA sequences being considerably divergent both within and without the group.

How can an organism manage to replicate a viable genome with such an accelerated rate of mutation? Woese et al. (1985) maintain that because a mollicute has a smaller genome, the entire genome can be replicated with the same overall accuracy as an organism that has a larger genome with a lower mutation rate (per base pair). Woese et al. (1985) propose that the mollicutes have a permanently accelerated rate of mutation and thus are in a state of macroevolution. Macroevolution as it is understood in the *Metazoa* is episodic; in the mollicutes, however, it continues unmitigated. Thus, the mollicutes have uniquely extreme phenotypes and are relatively heterogeneous among themselves (Woese et al., 1985).

Sladek (1986) proposed a three-step model for the evolution of the mollicutes from walled bacteria. First, as has been observed to occur naturally, L-forms arose from walled bacteria. Second, L-forms from the same or different species fused, creating polyploid entities. Such phenomena have been observed in the laboratory. Finally, through recombination between nontandem homologous sequences of the two genomes, substantial genome reductions occurred. It is well-established that

sequences with a high degree of homology are subject to homologous recombination in prokaryotes (Petes and Hill, 1988). Sladek does not address the question of whether this model of degenerative evolution of walled bacteria occurred only in the past or whether it continues today.

ENZYMIC ACTIVITIES OF CARBOHYDRATE, PURINE, AND PYRIMIDINE METABOLISM IN THE *ANAEROPLASMASTACEAE* (CLASS *MOLLICUTES*)

James P. Petzel[1], M. C. McElwain[2], D. DeSantis[2], J. Manolukas[2], M. V. Williams[2], P. A. Hartman[1], M. J. Allison[3], and J. D. Pollack[2]

[1]Department of Microbiology
Iowa State University, Ames, IA 50011-3211

[2]Department of Medical Microbiology and Immunology
The Ohio State University, Columbus, OH 43210-1239

[3]National Animal Disease Center
U.S.D.A. Agricultural Research Service, Ames, IA 50010

The senior author was a recipient of an American Society for Microbiology President's Travel Fellowship

SECTION I. ENZYMIC ACTIVITIES OF CARBOHYDRATE, PURINE, AND PYRIMIDINE METABOLISM IN THE *ANAEROPLASMATACEAE* (CLASS *MOLLICUTES*)

Abstract

Cell-free extracts of two strictly anaerobic mollicutes, *Anaeroplasma intermedium* 5LA and *Asteroleplasma anaerobium* 161^T, were tested for enzymic activities of intracellular carbohydrate metabolism. *As. anaerobium* was also tested for enzymes of purine and pyrimidine metabolism. Both organisms had enzymic activities associated with the nonoxidative portion of the pentose phosphate pathway, and with the Embden-Meyerhoff-Parnas pathway. The 6-phosphofructokinase (PFK) of *As. anaerobium* was ATP-dependent, whereas the PFK of *An. intermedium* was pyrophosphate-dependent (PP_i-dependent). The two anaerobic mollicutes also differed with respect to the enzymes that converted phospho-*enol*pyruvate (PEP) to pyruvate; *An. intermedium* had pyruvate kinase activity, but *As. anaerobium* had pyruvate, orthophosphate dikinase activity (PP_i-dependent). Both organisms had lactate dehydrogenase activity that was activated by fructose 1,6-bisphosphate (Fru-1,6-P_2). *An. intermedium* had activity for PEP carboxykinase (activated by Fru-1,6-P_2), but *As. anaerobium* did not. PEP carboxytransphosphorylase activity was not detected in either organism. *An. intermedium* had malate dehydrogenase and isocitrate dehydrogenase activities, but it had no activities for the three other tricarboxylic acid cycle enzymes examined; *As. anaerobium* had malate dehydrogenase activity only. *As. anaerobium* had enzymic activities for the interconversion of purine bases,

(deoxy)ribonucleosides, and (deoxy)ribomononucleotides, including PP_i-dependent nucleoside kinase, reported heretofore only in some other mollicutes. *As. anaerobium* could synthesize dTDP by the thymine salvage pathway if deoxyribose 1-phosphate was provided, and it had dUTPase, ATPase, and dCMP kinase activities. It lacked (deoxy)cytidine deaminase, dCMP deaminase, and deoxycytidine kinase activities.

Introduction

The wall-less eubacteria of the Class *Mollicutes* have come under increased study because of several interesting attributes. Because of the small size of their cells and genomes, it has been suggested that some mollicutes could serve as model organisms in the search for the minimal genetic and metabolic requirements of independent, self-replicating cells (Morowitz, 1984). Some of the carbohydrate, purine, pyrimidine, arginine, and urea metabolism and membrane physiology has been described for certain *Mollicutes* (Cocks et al., 1985, 1988; DeSantis et al., 1989; Manolukas et al., 1988; McElhaney, 1986; McElwain et al., 1988; Pollack et al., 1983; Romano et al., 1986). However, the intracellular metabolism of the *Anaeroplasmatales,* one of the three orders of the *Mollicutes,* has been neglected. The only study of the intracellular metabolism of a member of the *Anaeroplasmatales* is that of McElwain et al. (1988), who reported on enzymic activities of purine and pyrimidine metabolism in *Anaeroplasma intermedium* 5LA.

The *Anaeroplasmatales* are most prominently distinguished from the *Mycoplasmatales* and the *Acholeplasmatales* by the stable and absolute sensitivity of the anaerobic mollicutes to molecular oxygen and by the sequence data of the 5S rRNA of *Anaeroplasma* spp. (Robinson, 1979; Rogers et al., 1985). All isolates obtained thus far have been from the rumens of livestock. Before 1987, the only recognized genus in this group was *Anaeroplasma,* which included sterol-requiring strains with a G+C ratio of 29-34 mol%. When Robinson and Freundt (1987) elevated the strictly anaerobic mollicutes to the status of an order, they created a second genus, *Asteroleplasma. Asteroleplasma* are strictly anaerobic, sterol-nonrequiring strains with a G+C ratio of 40 mol%.

In the present study, we examined cytoplasmic extracts of a strain of *Anaeroplasma* and of *Asteroleplasma* for enzymic activities associated with intracellular carbohydrate metabolism (the pentose phosphate and Embden-Meyerhof-Parnas pathways, and the tricarboxylic acid cycle). We also tested the *Asteroleplasma* strain for enzymic activities associated with the interconversion of purine and pyrimidine bases, (deoxy)ribonucleosides, and (deoxy)ribomononucleotides.

The anaerobic mollicutes are the least-studied members of the Class *Mollicutes,* and this work is the first report of their intracellular carbohydrate metabolism. These results, and those dealing with purine and pyrimidine metabolism, may also be useful in clarifying the taxonomic divisions within the family *Anaeroplasmataceae* and provide information that may eventually be useful in elucidating the phylogenetic

relationships of the *Anaeroplasmatales* to other members of the Class *Mollicutes*.

Materials and Methods

Strains

Anaeroplasma intermedium 5LA and *Asteroleplasma anaerobium* 161^T (=ATCC 27880^T) were isolated from the rumens of sheep and obtained from I.M. Robinson, the National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, IA.

Cultivation and extract preparation

The anaerobic mollicutes were grown in 800-ml batches of S-2 medium, a modification of Robinson's MM-10 medium (1979). A batch of S-2 medium was prepared by dissolving the following in 700 ml of distilled H_2O: BBL Phytone, 1.6 g; Brain Heart Infusion, 1.6 g; Difco Soytone, 0.8 g; yeast extract, 0.8 g; Difco Malt Extract, 1.2 g; Difco soluble starch, 2.0 g; maltose, 0.8 g; glucose, 0.4 g. The sugars were cell culture-tested products from Sigma. [More recently, we have found that not all lots of peptones and carbohydrates support good growth. Currently, we are substituting Phytone with Deltown Chemurgic papaic digest of soymeal (SE50M; Fraser, NY).] The medium was supplemented with minerals, volatile fatty acids, resazurin, and cysteine hydrochloride as specified by Robinson (1979), except that Na_2SO_4 was substituted for $(NH_4)_2SO_4$, and the cysteine was added before the medium was sterilized in an autoclave.

The pH of the medium was adjusted to 7.2 with approx. 5 ml of 7.5 *M*-NaOH. The medium was prepared anaerobically under CO_2 by the modified Hungate technique (Robinson, 1979). After sterilization, the medium was supplemented with 75 ml of 8% (w/v) Na2CO3 (sterile and under CO_2; addition before autoclaving caused caramelization) and 6.4 ml of a 95% (v/v) ethanolic solution of cholesterol (2.5 mg/ml; analytical grade) and soybean lecithin (22.5 mg/ml; Calbiochem 429415; other lecithin preparations tested were not satisfactory). The lipid solution was sterilized by filtration (Millipore SLGV025LS); final concentrations of the lipids in S-2 medium were 20 µg of cholesterol/ml and 180 µg of lecithin/ml. The medium was bubbled with CO_2 until the resazurin was once again colorless. The flasks were inoculated (<1% inocula) and incubated at 37° for 72±3 h (*An. intermedium)* or 96±3 h (*As. anaerobium)*. Contributing factors in increased cell yields in S-2 medium compared with other formulations were (data not shown): i) soybean-based peptones and malt extract. ii) a final concentration of 0.75% Na_2CO_3 (versus the usual 0.4% in other anaerobic media) and the concomitant rise in final pH from 6.7 to 7.0. iii) a final concentration of 0.8% ethanol; delivery of the lipids in a smaller volume left the medium more opaque and less productive.

The cells were harvested by centrifugation and washed once with EW buffer: 15-m*M* PIPES (Na^+), 75-m*M* NaCl, 10-m*M* KCl, 1-m*M* $MgCl_2$, pH 7.0. The buffer was sterilized in an autoclave, cooled while bubbling with N_2, and dithiothreitol was added to 4 m*M* (sterilization was not necessary).

Cells were lysed osmotically in 1-m*M* dithiothreitol, 800-µ*M* NaCl, and 50-µ*M* HEPES, pH 7.4, or by explosive decompression in a Parr-Bomb. Lysates were clarified by centrifugation at 8,800 g_{max} at 4°C for 15 min and then again at 235,000 g_{max} at 4°C for 1 h.

Enzyme assays

Anaerobic precautions were taken where specifically indicated (see below). Enzymic reactions of the pentose phosphate pathway, the Embden-Meyerhof-Parnas (EMP) pathway, and the tricarboxylic acid (TCA) cycle were assayed by following the appearance or disappearance of substrates or cofactors spectrophotometrically. Methods used for enzymes of the pentose phosphate and EMP pathways are as follows. For polyphosphate-glucose phosphotransferase (EC 2.7.1.63), the reaction mixture of Szymona et al. (1967), containing polyphosphate (average of 25 residues; Sigma S 6128), was used; activity was detected enzymatically by the method of Szymona and Szymona (1979). The phosphorylation of glucose by carbamoyl phosphate (glucose 6-phosphatase, EC 3.1.3.9) was determined by the method of Lueck and Nordlie (1970). Other enzymes were assayed using the modifications specified by DeSantis et al. (1989) of the following methods: glucose-6-phosphate (Glc-6-*P*) dehydrogenase (EC 1.1.1.49) by the method of DeMoss (1955) (see also anaerobic method below); phosphogluconate dehydrogenase (EC 1.1.1.44) by the method of DeMoss (1955); ribulose-phosphate 3-epimerase (EC 5.1.3.1) and ribose-5-phosphate isomerase (EC 5.3.1.6) by the method of Horecker et al. (1957);

transketolase (EC 2.2.1.1), with ribose-5-phosphate (Rib-5-*P*) as the acceptor substrate, by method B of Kochetov (1982) and, with erythrose-4-phosphate (Ery-4-*P*) as the acceptor substrate, by the method of DeSantis et al. (1989); transaldolase (EC 2.2.1.2) in the forward direction by the method III of Venkataraman and Racker (1961) and in the reverse direction by the method of Venkataraman and Racker (1961); deoxyribose-phosphate aldolase (EC 4.1.2.4) by the method of Racker (1955); hexokinase (EC 2.7.1.1) by the method of Chou and Wilson (1975); glucose 6-phosphate isomerase (EC 5.3.1.9) by the method of Gracy and Tilley (1975); fructose-bisphosphate aldolase (EC 4.1.2.13) by the method of Taylor (1955); phosphoglycerate kinase (EC 2.7.2.3) by the method of Bücher (1955); phosphoglycerate mutase (EC 5.4.2.1) by the method of DeSantis et al. (1989); enolase (EC 4.2.1.11) by the method of Morse et al. (1974); ATP-dependent 6-phosphofructokinase (ATP-PFK; EC 2.7.1.11) by the method of Ling et al. (1955); and PP_i-dependent 6-phosphofructokinase (PP_i-PFK; EC 2.7.1.90) in both the forward (see also anaerobic method below) and the reverse directions by the methods of O'Brien et al. (1975).

The following enzymes associated with the TCA cycle were assayed by the methods used by Manolukas et al. (1988): pyruvate kinase (EC 2.7.1.40); malate synthase (EC 4.1.3.2); malate dehydrogenase [EC 1.1.1.37; the reverse direction was also assayed by using the method of Bergmeyer and Bernt (1983)]; aspartate aminotransferase (EC 2.6.1.1); fumarate hydratase (EC 4.2.1.2); aconitate hydratase (EC 4.2.1.3); and isocitrate dehydrogenase (EC 1.1.1.42). Other enzymes were assayed by

the following methods: pyruvate, orthophosphate dikinase (PPDK; EC 2.7.9.1) by the method of Ernst et al. (1986), assaying for the formation of ATP [we omitted $(NH_4)_6Mo_7O_{24}$] and by the method of Evans and Wood (1971), assaying for the formation of pyruvate; lactate dehydrogenase (LDH; EC 1.1.1.27) in the direction of pyruvate reduction by the method of Turunen et al. (1987) and in the direction of D(-)- or L(+)-lactate oxidation by the method of Neimark and Tung (1973) using the anaerobic conditions described below; pyruvate carboxylase (EC 6.4.1.1) by the method of Young et al. (1969); phospho*enol*pyruvate (PEP) carboxykinase (EC 4.1.1.49) by the method of Chang and Lane (1966) [we substituted dithiothreitol for glutathione and tested activity in the presence and absence of 0.5 m*M* fructose-1,6-bisphosphate (Fru-1,6-P_2)]; PEP carboxytransphosphorylase (EC 4.1.1.38) by the method of Wood et al. (1969) (we reduced the concentration of $CoCl_2$ to 10 μ*M*); pyruvate dehydrogenase (EC 1.2.4.1) by the method of Visser et al. (1982); and citrate synthase (EC 4.1.3.7) by methods 1 and 3 of Gottschalk and Dittbrenner (1970) (see also the anaerobic method below).

The assays of McElwain and Pollack (1987) (using radioisotopically labelled substrates and thin-layer chromatography) were used to examine the following enzymes associated with purine interconversions: phosphoribosyltransferases (EC 2.4.2.7 and -.8); purine-nucleoside phosphorylase (EC 2.4.2.1); 5'-nucleotidase (EC 3.1.3.5); and ATP-dependent nucleoside kinases (EC 2.7.1.20, -.73, and -.76; in these reactions, we also substituted PP_i for ATP).

The methods for assaying the following enzymic activities associated with pyrimidine metabolism, determined by using radioisotopically labelled substrates, have been described previously by Williams and Pollack (1985): dUMPase (EC 3.1.3.5); dCMP deaminase (EC 3.5.4.12); dCMP kinase (EC 2.7.4.14); dUTPase (EC 3.6.1.23); dTMP kinase (EC 2.7.4.9); thymidine kinase (EC 2.7.1.21); thymidine phosphorylase (EC 2.4.2.4); ATPase [EC 3.6.1.8; determined by substituting ATP for dUTP in the assay for dUTPase]; and cytidine deaminase [E.C. 3.5.4.5; determined by substituting cytidine (Cyd) for deoxycytidine (dCyd) in the deoxycytidine deaminase assay]. Deoxycytidine kinase (E.C. 2.7.1.74) activity was assayed by the method used by McElwain et al. (1988).

Anaerobic enzyme assays

Three enzymes that were not detected in the present study under aerobic conditions were also examined under anaerobic conditions; in addition, the oxidation of lactate by LDH was assayed (see above) only under anaerobic conditions. We assayed for Glc-6-*P* dehydrogenase by the method of Ujita and Kimura (1982), PP_i-PFK in the forward direction by the method of O'Brien et al. (1975), and citrate synthase by method 3 of Gottschalk and Dittbrenner (1970) using Ellman's reagent, with anaerobic precautions for all three assays as follows. Cell-free extracts contained dithiothreitol (1 m*M*) and were frozen under Ar. Except for the citrate synthase assay, all buffers were supplemented with dithiothreitol to 0.25 m*M*. All buffers were boiled while purging with Ar to drive off

dissolved O_2, cooled, and stored under Ar. Assays were conducted under Ar in spectrophotometric cuvettes with septum-screw caps (Thomas Scientific # 8493-D12). Reagents were withdrawn from stock solutions and introduced into the cuvette either while flushing with Ar or by syringe.

Chemicals

Sources of the radioisotopically labelled substrates for purine and pyrimidine studies have been described elsewhere (McElwain and Pollack, 1987; McElwain et al., 1988; Williams and Pollack, 1985). Biochemicals were obtained from Sigma Chemical Company unless specified otherwise. Protein concentrations were determined by using the Bio-Rad protein assay reagent with bovine serum albumin as the standard.

Results

Pentose phosphate pathway

Cell-free extracts of both *Anaeroplasma intermedium* 5LA and *Asteroleplasma anaerobium* 161^T did not contain detectable glucose-6-phosphate (Glc-6-*P*) dehydrogenase (assayed aerobically or anaerobically) or 6-phosphogluconate dehydrogenase activities (Table 1). However, both organisms did have other enzyme activities of this pathway: ribulose-phosphate 3-epimerase, Rib-5-*P* isomerase, transketolase, and transaldolase. Both organisms also had activity for deoxyribose-phosphate aldolase.

Glycolysis

Both anaerobic mollicutes possessed intracellular enzymic activities associated with the Embden-Meyerhof-Parnas (EMP) pathway for the metabolism of glucose, including that of the marker enzyme of this pathway, 6-phosphofructokinase (PFK) (Table 2). The steps from glucose to the triose phosphates and from 3-phosphoglyceric acid to pyruvate were examined. Cell extracts of *An. intermedium* exhibited enzymic activities at all eight steps. *As. anaerobium* cell-free extracts exhibited activities at all steps of the EMP pathway examined except hexokinase [ATP, PP_i, polyphosphate, and PEP were tested as phosphate donors in the hexokinase reaction, without effect. PEP was also inactive as a phosphate donor with resuspended membrane fraction (data not shown)]. In addition, *As. anaerobium* had no carbamoyl phosphate-dependent activity for the phosphorylation of glucose by glucose 6-phosphatase. The hexokinase of *An. intermedium* was ATP-dependent.

The PFK activity of *An. intermedium* was PP_i-dependent, whereas the PFK of *As. anaerobium* was active only with ATP. To ensure that ATP solutions were not contaminated with PP_i, which could yield spurious results with PP_i-PFK (Reeves, 1987), ATP solutions were assayed for PP_i with a commercial kit (Sigma), and none was detected.

The tricarboxylic acid cycle and associated enzymes

An. intermedium had activity for pyruvate kinase, converting PEP to pyruvate while concomitantly converting ADP to ATP (Table 3). *As.*

anaerobium had no activity for pyruvate kinase. Instead, *As. anaerobium* had activity for pyruvate, orthophosphate dikinase, thereby converting PEP, AMP, and PP_i to pyruvate, ATP, and P_i.

The anaerobic mollicutes had limited enzymic activities of the TCA cycle (Table 3). Both organisms had malate dehydrogenase and aspartate aminotransferase activities. No activities were detected in extracts of *As. anaerobium* for any of the following enzymes of the TCA cycle that were examined (Table 3): isocitrate dehydrogenase, fumarase, citrate synthase, and aconitase. *An. intermedium* was also negative for all these enzymes except isocitrate dehydrogenase; this enzyme was active in the absence of added Mn^{++}.

An. intermedium and *As. anaerobium* had lactate dehydrogenase (LDH) activity in the direction of pyruvate reduction, and the activities of both species were increased by Fru-1,6-P_2 (data not shown). We could not detect activity in either organism for D(-)- or L(-)-lactate oxidation by LDH with either NAD^+ or its 3-acetylpyridine analog.

An. intermedium (but not *As. anaerobium)* had activity for PEP carboxykinase; the activity was dependent on the presence of both ADP (or GDP) and Fru-1,6-P_2. Additionally, the enzyme was active when assayed at pH 6.6 (100-m*M* imidazole), but not at pH 7.8 (50-m*M* Tris). Both organisms lacked detectable activities for PEP carboxytransphosphorylase and pyruvate dehydrogenase. *An. intermedium* had pyruvate carboxylase activity but *As. anaerobium* did not.

Purine metabolism of *As. anaerobium*

Extracts of *As. anaerobium* had enzymic activities for purine metabolism that would enable it to synthesize (deoxy)ribomononucleotides from purine bases in either of two ways (Table 4). In one instance, phosphoribosyltransferases converted phosphoribosylpyrophosphate and the bases adenine, hypoxanthine, or guanine directly to the respective ribomononucleotides, AMP, IMP, or GMP. Alternatively, in a two-step process, nucleoside phosphorylase could convert ribose-1-phosphate (Rib-1-*P*) or deoxyribose-1-phosphate (dRib-1-*P*) and the bases adenine, hypoxanthine, or guanine to the respective (deoxy)ribonucleosides, (deoxy)adenosine, (deoxy)inosine, or (deoxy)guanosine; then, PP_i-dependent (deoxy)ribonucleoside kinase(s) converted the (deoxy)nucleosides, (deoxy)adenosine, inosine, or guanosine, to the respective (deoxy)ribomononucleotides, (d)AMP, IMP, or GMP. No ATP-dependent (deoxy)ribonucleoside kinase activity was detected in extracts of *As. anaerobium*. Extracts of *As. anaerobium* also had 5'-nucleotidase activity. Finally, *As. anaerobium* had deoxyribomononucleotidase activity.

Pyrimidine deoxyribonucleotide metabolism of *As. anaerobium*

Extracts of *As. anaerobium* possessed thymidine phosphorylase, thymidine kinase, and dTMP kinase activities (Table 5). *As. anaerobium* also possessed both dUTPase and ATPase activities. This organism lacked dUMPase, dCMP deaminase, deoxycytidine kinase and (deoxy)cytidine

deaminase (tested with Cyd as the substrate) activities, but it possessed dCMP kinase activity.

Discussion

The method used for each enzyme assay of extracts of the anaerobic mollicutes was not optimized. Thus, the data presented here are more useful in a qualitative than a quantitative sense, especially in regard to drawing conclusions about rates of activities *in vivo*. Where possible, enzymes from commercial sources or control organisms were used to confirm the efficacy of the assays when no activity was detected in extracts of the anaerobic mollicutes.

Examination of the literature reveals that some enzymes are O_2-labile regardless of the organism under study (i.e., nitrogenase, formate acetyltransferase). However, the literature indicates that enzymes of central metabolic pathways common to both aerobic and anaerobic organisms are usually not O_2-labile. Although some researchers assay all such enzymes from an anaerobic bacterium under anaerobic conditions (Hoshino et al., 1978), other researchers have detected activities for such glycolytic enzymes from a variety of anaerobic bacteria without anaerobic conditions [the studies of Joyner and Baldwin (1966) of six genera of ruminal bacteria being but one such example]. In the present study, most enzymes were assayed without anaerobic precautions because these enzymes have not been reported to be O_2-labile in other bacteria. However, we did assay Glc-6-*P* dehydrogenase, PP_i-PFK, and citrate synthase both

aerobically and anaerobically. Assays conducted anaerobically did not reveal any activities that were not observed under aerobic conditions.

The physiology and enzymic activities of the *Anaeroplasmataceae* are similar in many respects to those reported for the *Mycoplasmatales* and the *Acholeplasmatales* (Cocks et al., 1985, 1988; DeSantis et al., 1989; Manolukas et al., 1988; McElwain and Pollack, 1987; McElwain et al., 1988), but there are some distinctions. In the anaerobic mollicutes, the hexose monophosphate shunt was not active as such; both *An. intermedium* and *As. anaerobium* lacked the oxidative enzymes of the pathway, Glc-6-*P* dehydrogenase and 6-phosphogluconate dehydrogenase. However, both anaerobic mollicutes had activities for the remaining enzymes of the pentose phosphate pathway. Thus, the *Anaeroplasmataceae* have an enzyme pattern for the pentose phosphate pathway similar to that of *Mycoplasma* spp. and different from *Acholeplasma* spp., which have activities for the two oxidative enzymes (DeSantis et al., 1989). The presence of some of the enzymes of this pathway suggests that the anaerobic mollicutes may interconvert sugar phosphates and carbon skeletons of the EMP pathway and various other biosynthetic routes. Cocks et al. (1985) and DeSantis et al. (1989) proposed that deoxyribose-phosphate aldolase activity links nucleic acid and carbohydrate metabolism.

Several extract preparations had no activity for transaldolase. Such variability in transaldolase activity has been reported for other mollicutes (Cocks et al., 1985; DeSantis et al., 1989). Cocks et al. (1985) reported that commercial enzyme preparations used as coupling

enzymes had an inhibitory effect on transaldolase activity in *Mycoplasma mycoides* subsp. *mycoides*.

Although the lack of both the oxidative enzymes of the pentose phosphate pathway and the lack of the concomitant generation of NADPH by *Mycoplasma* spp. may explain their increased need for exogenous lipids as compared with *Acholeplasma* spp. (DeSantis et al., 1989), the results in the present study do not make the same distinction between the sterol-requiring and the sterol-nonrequiring anaerobic mollicutes.

Both anaerobic mollicutes had enzymic activities associated with the EMP pathway, and this is probably their major route of glucose catabolism. *An. intermedium* possessed activities for all eight enzymes of this pathway, and *As. anaerobium* had activities for all these enzymes except hexokinase. Our inability to detect hexokinase activity (or any enzyme) does not demonstrate unequivocably that the organism indeed lacks the enzyme.

Our most noteworthy observation in studies of the EMP pathway was related to phosphofructokinase (PFK) activity. The PFK of *As. anaerobium* was ATP-dependent, as in *Mycoplasma* spp. (DeSantis et al., 1989) and most bacteria, whereas the PFK of *An. intermedium* was PP_i-dependent. Among bacteria, PP_i-PFK has been reported heretofore in *Acholeplasma* spp. (DeSantis et al., 1989; Pollack and Williams, 1986), *Deleya* spp. (Baumann et al., 1983), *Propionibacterium freundenreichii* and *P. acidipropionici* ("*P. arabinosum*"), some *Bacteroides* spp. and a *Clostridium* sp. (Wood, 1985). Thus, the correlation between sterol requirement and the type of

PFK in the *Anaeroplasmataceae* is the converse of that among the other mollicutes. PP_i is a putative evolutionary precursor of ATP (Kulaev and Vagabov, 1983; Wood, 1985); thus, the possession of a PP_i-dependent kinase by an organism may represent the retention of a phylogenetically earlier form of the enzyme.

The possession of a PP_i-PFK by an anaerobe also has physiological significance. Because most anaerobic heterotrophs cannot carry out oxidative phosphorylation, their generation of ATP is limited to a few substrate-level phosphorylation reactions. However, these anaerobes cannot regenerate NAD^+ by oxidative phosphorylation, so they must balance ATP-yielding reactions (i.e., acetate kinase) with NADH-oxidizing reactions (i.e., lactate dehydrogenase) such that there is sufficient NAD^+ for glycolysis to continue (Holland et al., 1987; Morris, 1985). The presence of PP_i-linked kinases, such as PP_i-PFK, may reduce the utilization of ATP in anaerobes (Wood, 1985); *An. intermedium* may conserve ATP in this manner.

The two anaerobic mollicutes can also be differentiated by the enzymes responsible for the conversion of PEP to pyruvate at the last step of glycolysis. *As. anaerobium* has PP_i-dependent pyruvate, orthophosphate dikinase (PPDK) activity, whereas *An. intermedium* possesses the more common pyruvate kinase, which is present in all other mollicutes examined (Manolukas et al., 1988). PPDK has been found in only a few other bacteria: *Clostridium symbiosum, P. freundenreichii, P. acidipropionici, Acetobacter* spp., a clostridium isolated from the

cockroach gut, and three species of anoxygenic photosynthetic bacteria (Ernst et al., 1986; Evans and Wood, 1971; Wood, 1985). The acetobacters and *P. freundenreichii* have both PPDK and pyruvate kinase. It has been suggested that PPDK is active in these bacteria in the direction of PEP for gluconeogenesis or in the formation of oxaloacetic acid, and that pyruvate kinase is active in the direction of pyruvate during glycolysis (Evans and Wood, 1971; Wood, 1985). In contrast, *C. symbiosum* (Evans and Wood, 1971; Wood, 1985) and *As. anaerobium* lack pyruvate kinase, and thus, PPDK is probably active in the direction of pyruvate during glycolysis.

Enzymic activities of the anaerobic mollicutes in the TCA cycle were limited. Both organisms had activity for malate dehydrogenase, but of the four other TCA-cycle enzymes examined, there was activity only for isocitrate dehydrogenase (ICDH) in extracts of *An. intermedium*. Because *An. intermedium* had no detectable citrate synthase or aconitase activities, the ICDH activity was not linked to the rest of the TCA cycle in typical fashion. However, α-ketoglutarate, a reactant of the ICDH reaction, is also a reactant in the aspartate aminotransferase reaction, for which *An. intermedium* had activity. In contrast, *As. anaerobium* lacked ICDH activity, and thus, is similar to the eight *Mycoplasma* strains examined by Manolukas et al. (1988), which lacked detectable activity for all TCA cycle enzymes except malate dehydrogenase.

Only *An. intermedium* was able to carboxylate PEP to oxaloacetate, but both organisms had LDH activity, reducing pyruvate to lactate [one of

the fermentation products of *Anaeroplasma* spp. (Robinson, 1979)], and thereby generating NAD^+ for use in the EMP pathway. We could not detect the oxidation of lactate by LDH in either organism (tested with NAD^+ and its 3-acetylpyridine analog). Neimark and Tung (1973) detected L(+)-lactate oxidation in extracts of *Acholeplasma laidlawii* with the 3-acetylpyridine analog, but not with NAD^+. Turunen et al. (1987) detected LDH activity in extracts of *Clostridium thermohydrosulfuricum* spectrophotometrically in the direction of pyruvate reduction; L(+)-lactate oxidation LDH activity was detected with a gel-staining method but could not be detected spectrophotometrically.

As. anaerobium had enzymic activities for the interconversion of purine bases, (deoxy)ribonucleosides, and (deoxy)ribomononucleotides; such activities could be important in the salvage pathway for purines. The occurrence of PP_i-dependent (deoxy)ribonucleoside kinase among both *An. intermedium* (McElwain et al., 1988) and *As. anaerobium* (this study), heretofore reported only in some other *Mollicutes* (McElwain and Pollack, 1987), represents another reaction in which the energy of a PP_i bond is utilized, thereby conserving ATP.

As. anaerobium is capable of synthesizing dTDP by the thymine salvage pathway, provided that there is a source of dRib-1-*P*. But *As. anaerobium* cannot use either dCyd or dCMP as a source of dRib-1-*P* because it lacks both dCMP deaminase and (deoxy)cytidine deaminase (tested with Cyd as the substrate) activities. This is in contrast to *An. intermedium*, which possesses both these enzymic activities (McElwain et

al., 1988; Williams and Pollack, unpublished result). Neale et al. (1983) reported that *M. mycoides* subsp. *mycoides* used dCMP as a source of dRib-1-*P* after deamination and derobosylation. It should be pointed out that although Finch and his co-workers have not been able to detect dCyd deaminase activity in extracts of *M. mycoides* subsp. *mycoides*, they do have evidence suggesting that the activity exists *in vivo* (Cocks et al., 1988).

In addition to ostensibly being unable to utilize dCyd as a source of dRib-1-*P* for thymidine biosynthesis, we propose that *As. anaerobium* cannot use dCyd for the salvage synthesis of dCyd nucleotides because it lacks detectable dCyd kinase activity. Of those mollicutes that have been examined, the lack of dCyd kinase activity is unique to the anaerobic mollicutes (McElwain et al., 1988; this study). Neale et al. (1984) reported that *M. mycoides* subsp. *mycoides* incorporates deoxyribonucleoside monophosphates directly from the growth medium and subsequently phosphorylates them to the triphosphate form. An analogous system may be present in the anaerobic mollicutes because both *An. intermedium* (Williams and Pollack, unpublished result) and *As. anaerobium* have dCMP kinase activity.

The results reported here represent an important first step in elucidating the intracellular physiology of the *Anaeroplasmataceae*, the least-studied members of the *Mollicutes*. These anaerobic mollicutes have both important similarities to and differences with each other and other mollicutes. Further studies should be conducted to determine if these findings extend to the other three species of the genus *Anaeroplasma*.

The present and previous physiological investigations of the *Mollicutes* have contributed to our knowledge of these bacteria, but the physiology of *Mollicutes* has not been described in its entirety. Future physiological investigations may contribute to further clarification of the phylogenetic relationships among the *Mollicutes* and allied walled bacteria.

Table 1. Enzymes of the pentose phosphate pathway in cell-free extracts of strictly anaerobic mollicutes[a]

Enzyme	*Anaeroplasma intermedium*	*Asteroleplasma anaerobium*
Glucose-6-phosphate dehydrogenase	NA[b]	NA
Phosphogluconate dehydrogenase	NA	NA
Ribulose-phosphate 3-epimerase	6.4±3.0 4.9±1.8	7.0±3.6 12±9.8
Ribose-5-phosphate isomerase	5.7±2.2 1.4±2.2	25±13 13±5.4
Transketolase[c]	18±13 10±7.0	9.7±8.1 3.5±3.0
Transaldolase	0.5±1.0 NA	3.4±4.1 NA
Deoxyribose-phosphate aldolase	1.7±1.5	0.3±0.5

[a]Nanomoles of product formed per minute per mg protein (± S.D.; average of three batches of cells). See text for details of assays. For each enzyme, the first line of data refers to activities in the forward direction (as listed in Webb, 1984); the second line (if present) refers to activities in the reverse direction.

[b]NA, no activity detected.

[c]The first line refers to the reaction with Rib-5-*P* as the acceptor substrate, the second line, with Ery-4-*P* as the acceptor substrate.

Table 2. Enzymes of the Embden-Meyerhof-Parnas pathway in cell-free extracts of strictly anaerobic mollicutes[a]

Enzyme	*Anaeroplasma intermedium*	*Asteroleplasma anaerobium*
Hexokinase	220	NA[b]
Polyphosphate-glucose phosphotransferase	NA	NA
Glucose 6-phosphatase (carbamoyl-*P*)[c]	NA	NA
Glucose-6-phosphate isomerase	250 450	5.7±2.3 200±130
6-Phosphofructokinase (PP_i) (ATP)	49±19 220 NA	NA NA 7.7±3.8
Fructose-biphosphate aldolase	20±6.7	38±12
Phosphoglycerate kinase	1200	260±13
Phosphoglycerate mutase[c]	3.4	590±72
Enolase	31	630±210

[a]Nanomoles of product formed per minute per mg protein (± S.D. if number of batches of cells > 2; no S.D. given if number = 2). See text for details of assays. Except as noted (footnote c), the first line of data for each enzyme refers to activities in the forward direction (as listed in Webb, 1984); the second line (if present), refers to activities in the reverse direction.

[b]NA, no activity detected.

[c]Data refer to activities in the reverse direction (as listed in Webb, 1984).

Table 3. Enzymes associated with the tricarboxylic acid cycle in cell-free extracts of strictly anaerobic mollicutes[a]

Enzyme	*Anaeroplasma intermedium*	*Asteroleplasma anaerobium*
Pyruvate kinase	5.2±0.6 30±3.4	NA[b] NA
Pyruvate, orthophosphate dikinase[c]	NA	210
Lactate dehydrogenase	NA 77±4.7	NA 40±25
Pyruvate decarboxylase	2.5±0.3	NA
PEP carboxykinase[c]	141±31	NA
PEP carboxytransphosphorylase[c]	NA	NA
Pyruvate dehydrogenase	NA	NA
Malate synthase	NA	NA
Malate dehydrogenase	450±21 76±4.3	16 NA
Aspartate aminotransferase	37±4.0 6.8±0.3	4800±2100 33±16
Isocitrate dehydrogenase	41±31	NA
Fumarate hydratase	NA	NA
Citrate synthase[c]	NA	NA
Aconitate hydratase	NA	NA

[a]Nanomoles of product formed per minute per mg protein (± S.D. if number of batches of cells > 2). See text for details of assays. Except where noted (footnote c), the first line of data refers to activities in the forward direction (as listed in Webb, 1984); the second line (if present), refers to activities in the reverse direction.

[b]NA, no activity detected; PEP, phospho*enol*pyruvate.

[c]Data refer to activities in the reverse direction (as listed in Webb, 1984).

Table 4. Enzymes of purine metabolism in cell-free extracts of *Asteroleplasma anaerobium*

Enzyme (substrate)	picomoles formed per minute per mg protein[a]
Phosphoribosyltransferase	
adenine	1.1±0.1
hypoxanthine	1.4±0.4
guanine	1.0±0.4
Nucleoside phosphorylase	
adenine + Rib-1-*P*[b] ——> adenosine	5.6±0.6
adenine + dRib-1-*P* ——> deoxyadenosine	8.0±1.4
hypoxanthine + Rib-1-*P* ——> inosine	8.8±2.1
hypoxanthine + dRib-1-*P* ——> deoxyinosine	5.4±1.0
guanine + Rib-1-*P* ——> guanosine	9.0±2.5
guanine + dRib-1-*P* ——> deoxyguanosine	19±9.3
Nucleoside kinase (PP_i- or ATP-linked)	
adenosine	0.8±0.2/NA
deoxyadenosine	0.5±0.1/NA
inosine	0.5±0.3/NA
guanosine	1.1±0.3/NA
deoxyguanosine	NA/NA
5'Nucleotidase	
AMP	3.0±0.5
dAMP	5.2±0.7
IMP	780±230
GMP	8.3±0.6

[a]Data are the means of determinations from three batches of cells ± S.D. See text for details of assays.

[b]Rib-1-*P*, ribose-1-phosphate; dRib-1-*P*, deoxyribose-1-phosphate; NA, no activity detected.

Table 5. Enzymes of pyrimidine metabolism in cell-free extracts of *Asteroleplasma anaerobium*

Enzyme	nanomoles formed per minute per mg protein[a]
Cytidine deaminase	NA[b]
dCMP deaminase	NA
Deoxycytidine kinase	NA
dCMP kinase	0.1±0.1
dTMP kinase	0.4±0.1
Thymidine kinase	0.3±0.1
Thymidine phosphorylase	17±0.4
dUMPase	NA
dUTPase	3.4±0.5
ATPase	3.1±0.3

[a]Data are the means of determinations from three batches of cells ± S.D. See text for details of assays. Data refer to activities in the forward direction (as listed in Webb, 1984).

[b]NA, no activity detected.

References Cited

Baumann, L., R. D. Bowditch, and P. Baumann. 1983. Description of *Deleya* gen. nov. created to accommodate the marine species *Alcaligenes aestus, A. pacificus, A. cupidus, A. venustus,* and *Pseudomonas marina.* Int. J. Syst. Bacteriol. 33:793-802.

Bergmeyer, H. U., and E. Bernt. 1983. Malate dehydrogenase: Oxaloacetate to malate reaction. Pages 171-175 *in* H. U. Bergmeyer, J. Bergmeyer, and M. Graßl (eds.), Methods of enzymatic analysis. Third edition. Volume III. Enzymes 1. Verlag Chemie, Weinheim.

Bücher, T. 1955. Phosphoglycerate kinase from brewer's yeast. Methods Enzymol. 1:415-422.

Chang, H.-C., and M. D. Lane. 1966. The enzymatic carboxylation of phosphoenolpyruvate. J. Biol. Chem. 241:2413-2420.

Chou, A. C., and J. E. Wilson. 1975. Hexokinase of rat brain. Methods Enzymol. 42:20-25.

Cocks, B. G., F. A. Brake, A. Mitchell, and L. R. Finch. 1985. Enzymes of intermediary carbohydrate metabolism in *Ureaplasma urealyticum* and *Mycoplasma mycoides* subsp. *mycoides.* J. Gen. Microbiol. 131:2129-2135.

Cocks, B. G., R. Youil, and L. R. Finch. 1988. Comparison of enzymes of nucleotide metabolism in two members of the *Mycoplasmataceae* family. Int. J. Syst. Bacteriol. 38:273-278.

DeMoss, R. D. 1955. Glucose-6-phosphate and 6-phosphogluconic dehydrogenases from *Leuconostoc mesenteroides.* Methods Enzymol. 1:328-334.

DeSantis, D., V. V. Tryon, and J. D. Pollack. 1989. Metabolism of mollicutes: The Embden-Meyerhof-Parnas pathway and the hexose monophosphate shunt. J. Gen. Microbiol. 135:683-691.

Ernst, S. M., R. J. A. Budde, and R. Chollet. 1986. Partial purification and characterization of pyruvate,orthophosphate (sic) dikinase from *Rhodospirillum rubrum.* J. Bacteriol. 165:483-488.

Evans, H. J., and H. G. Wood. 1971. Purification and properties of pyruvate phosphate dikinase from propionic acid bacteria. Biochemistry 10:721-729.

Gottschalk, G., and S. Dittbrenner. 1970. Properties of (*R*)-citrate synthase from *Clostridium acidi-urici.* Hoppe-Seyler's Z. Physiol. Chem. 351:1183-1190.

Gracy, R. W., and B. E. Tilley. 1975. Phosphoglucose isomerase of human erythrocytes and cardiac tissue. Methods Enzymol. 41:392-400.

Holland, K. T., J. S. Knapp, and J. G. Shoesmith. 1987. Anaerobic bacteria. Chapman and Hall, New York.

Horecker, B. L., J. Hurwitz, and P. K. Stumpf. 1957. The enzymatic synthesis of ribulose-1,5-diphosphate and xylulose-5-phosphate. Methods Enzymol. 3:193-195.

Hoshino, E., F. Frölander, and J. Carlsson. 1978. Oxygen and the metabolism of *Peptostreptococcus anaerobius* VPI4330-1. J. Gen. Microbiol. 107:235-248.

Joyner, A. E., Jr., and R. L. Baldwin. 1966. Enzymatic studies of pure cultures of rumen microorganisms. J. Bacteriol. 92:1321-1330.

Kochetov, G. A. 1982. Transaldolase from yeast, rat liver, and pig brain. Methods Enzymol. 90:209-223.

Kulaev, I. S., and V. M. Vagabov. 1983. Polyphosphate metabolism in micro-organisms. Adv. Microb. Physiol. 24:83-171.

Ling, K.-H., W. L. Byrne, and H. Lardy. 1955. Phosphohexokinase. Methods Enzymol. 1:306-31D.

Lueck, J. D., and R. C. Nordlie. 1970. Carbamyl phosphate:glucose phosphotransferase activity of hepatic microsomal glucose 6-phosphatase at physiological pH. Biochem. Biophys. Res. Commun. 39:190-196.

Manolukas, J. T., M. F. Barile, D. K. F. Chandler, and J. D. Pollack. 1988. Presence of anaplerotic reactions and transamination, and the absence of the tricarboxylic acid cycle in mollicutes. J. Gen. Microbiol. 134:791-800

McElhaney, R. N. 1986. Modifications of membrane lipid structure and their influence on cell growth, passive permeability, and enzymatic and transport activities in *Acholeplasma laidlawii* B. Biochem. Cell Biol. 64:58-65.

McElwain, M. C., and J. D. Pollack. 1987. Synthesis of deoxyribomononucleotides in mollicutes: Dependence on deoxyribose-1-phosphate and PP_i. J. Bacteriol. 169:3647-3653.

McElwain, M. C., D. K. F. Chandler, M. F. Barile, T. F. Young, V. V. Tryon, J. W. Davis, Jr., J. P. Petzel, C.-J. Chang, M. V. Williams, and J. D. Pollack. 1988. Purine and pyrimidine metabolism in *Mollicutes*. Int. J. Syst. Bacteriol. 38:417-423.

Morowitz, H. J. 1984. The completeness of molecular biology. Isr. J. Med. Sci. 20:750-753.

Morris, J. G. 1985. Anaerobic metabolism of glucose. Pages 357-378 *in* M. Moo-Young, A. T. Bull, and H. Dalton (eds.), Comprehensive biotechnology, the principles, applications and regulations of biotechnology in industry, agriculture and medicine. Pergamon Press, Oxford.

Morse, S. A., S. Stein, and J. Hines. 1974. Glucose metabolism in *Neisseria gonorrhoeae*. J. Bacteriol. 120:702-714.

Neale, G. A. M., A. Mitchell, and L. R. Finch. 1983. Enzymes of pyrimidine deoxyribonucleotide metabolism in *Mycoplasma mycoides* subsp. *mycoides*. J. Bacteriol. 156:1001-1005.

Neale, G. A. M., A. Mitchell, and L. R. Finch. 1984. Uptake and utilization of deoxynucleoside 5'-monophosphates by *Mycoplasma mycoides* subsp. *mycoides*. J. Bacteriol. 158:943-947.

Neimark, H., and M. C. Tung. 1973. Properties of a fructose-1,6-diphosphate-activated lactate dehydrogenase from *Acholeplasma laidlawii* type A. J. Bacteriol. 114:1025-1033.

O'Brien, W. E., S. Bowien, and H. G. Wood. 1975. Isolation and characterization of a pyrophosphate-dependent phosphofructokinase from *Propionibacterium shermanii*. J. Biol. Chem. 250:8690-8695.

Pollack, J. D., V. V. Tryon, and K. D. Beaman. 1983. The metabolic pathways of *Acholeplasma* and *Mycoplasma:* An overview. Yale J. Biol. Med. 56:709-716.

Pollack, J. D., and M. V. Williams. 1986. PPi-dependent phosphofructotransferase (phosphofructokinase) activity in the mollicutes (mycoplasma) *Acholeplasma laidlawii*. J. Bacteriol. 165:53-60.

Racker, E. 1955. Deoxyribose phosphate aldolase (DR-aldolase). Methods Enzymol. 1:384-386.

Reeves, R.E. 1987. Metabolic energy supplied by PP_i. Pages 255-259 *in* A. Torriani-Gorini, F. G. Rothman, S. Silver, A. Wright, and E. Yagil (eds.), Phosphate metabolism and cellular regulation in microorganisms. American Society for Microbiology, Washington, D.C.

Robinson, I. M. 1979. Special features of anaeroplasmas. Pages 515-528 *in* M. F. Barile and S. Razin (eds.), The mycoplasmas. Volume 1. Cell biology. Academic Press, Inc., New York.

Robinson, I. M., and E. A. Freundt. 1987. Proposal for an amended classification of anaerobic mollicutes. Int. J. Syst. Bacteriol. 37:78-81.

Rogers, M. J., J. Simmons, R. T. Walker, W. G. Weisburg, C. R. Woese, R. S. Tanner, I. M. Robinson, D. A. Stahl, G. Olsen, R. H. Leach, and J. Maniloff. 1985. Construction of the mycoplasma evolutionary tree from 5S rRNA sequence data. Proc. Natl. Acad. Sci. USA 82:1160-1164.

Romano, N., D. Russo Alesi, R. La Licata, and G. Tolone. 1986. Effects of urea phosphate (sic), ammonium ions and pH on *Ureaplasma* ATP synthesis. Microbiologica (Bologna) 9:405-413.

Szymona, O., and M. Szymona. 1979. Polyphosphate- and ATP-glucose phosphotransferase activities of *Nocardia minima*. Acta Microbiol. Pol. 28:153-160.

Szymona, O., S. O. Uryson, and I. S. Kulaev. 1967. Detection of polyphosphate glucokinase in various microorganisms. Biochemistry (Engl. Transl.) 32:408-415.

Taylor, J. F. 1955. Aldolase from muscle. Method Enzymol. 1:310-315.

Turunen, M., E. Parkkinen, J. Londesborough, and M. Korhola. 1987. Distinct forms of lactate dehydrogenase purified from ethanol- and lactate-producing cells of *Clostridium thermohydrosulfuricum*. J. Gen. Microbiol. 133:2865-2873.

Ujita, S., and K. Kimura. 1982. Glucose-6-phosphate dehydrogenase, vegetative and spore *Bacillus subtilis*. Methods Enzymol. 89:258-261.

Venkataraman, R., and E. Racker. 1961. Mechanism of action of transaldolase. I. Crystallization and properties of yeast enzyme. J. Biol. Chem. 236:1876-1882.

Visser, J., H. Kester, K. Jeyaseelan K, and R. Topp. 1982. Pyruvate dehydrogenase complex from *Bacillus*. Methods Enzymol. 89:399-407.

Webb, E. C. 1984. Enzyme nomenclature. Academic Press, Inc., Orlando.

Williams, M. V., and J. D. Pollack. 1985. Pyrimidine deoxyribonucleotide metabolism in *Acholeplasma laidlawii* B-PG9. J. Bacteriol. 161:1029-1033.

Wood, H. G. 1985. Inorganic pyrophosphate and polyphosphates as sources of energy. Curr. Top. Cell. Regul. 26:355-360.

Wood, H. G., J. J. Davis, and J. M. Willard. 1969. Phosphoenolpyruvate carboxytransphosphorylase from *Propionibacterium shermanii*. Methods Enzymol. 13:297-309.

Young, M. R., B. Tolbert, and M. F. Utter. 1969. Pyruvate carboxylase from *Saccharomyces cerevisiae.* Methods Enzymol. 13:250-258.

PYROPHOSPHATE-DEPENDENT ENZYMES IN WALLED BACTERIA PHYLOGENETICALLY RELATED TO THE WALL-LESS *MOLLICUTES*

James P. Petzel[1], Paul A. Hartman[1], and Milton J. Allison[2]

[1]Department of Microbiology
Iowa State University, Ames, IA 50011-3211

[2]National Animal Disease Center
U.S.D.A. Agricultural Research Service, Ames, IA 50010

SECTION II. PYROPHOSPHATE-DEPENDENT ENZYMES IN WALLED BACTERIA PHYLOGENETICALLY RELATED TO THE WALL-LESS *MOLLICUTES*

Abstract

Some of the wall-less bacteria of the class *Mollicutes* have pyrophosphate-dependent (PP_i-dependent) enzymic activities including: PP_i-phosphofructokinase (PP_i-PFK); PP_i-nucleoside kinase; and pyruvate, orthophosphate dikinase (PPDK). In most other bacteria, ATP, not PP_i, is the cofactor in analogous enzymic reactions. Because of the prominence of PP_i-dependent enzymes among the mollicutes, enzymes that occur in other bacteria infrequently, we report here the examination of the six walled bacteria reported to be phylogenetically related to the mollicutes (*Clostridium innocuum, Clostridium ramosum, Erysipelothrix rhusiopathiae, Lactobacillus catenaformis, Lactobacillus vitulinus,* and *Streptococcus pleomorphus*) for PP_i- and ATP-PFK, phospho*enol*pyruvate (PEP) carboxytransphosphorylase, PPDK, and PP_i- and ATP-acetate kinase. Two anaerobic mollicutes, *Anaeroplasma intermedium* and *Asteroleplasma anaerobium,* were tested also. *C. innocuum, E. rhusiopathiae, S. pleomorphus,* and *An. intermedium* had PP_i-PFK activities, whereas *C. ramosum,* the two lactobacilli, and *As. anaerobium* had only ATP-PFK activities. *As. anaerobium* and all the walled bacteria except *E. rhusiopathiae* had PPDK activities. All species except *As. anaerobium* and *E. rhusiopathiae* also had pyruvate kinase activities; the effects of allosteric activators were tested. PEP carboxytransphosphorylase was detected by two methods in *C. innocuum, C. ramosum,* and *S. pleomorphus.* All the species tested had ATP-acetate kinase activities, but none had

detectable PP_i-acetate kinase activities. The occurrence of one or more PP_i-dependent enzymes in the mollicutes and their walled relatives is a phenotypic indicator of their phylogenetic relatedness. The distribution of these enzymes among members of this group substantiates the subgroups as proposed by others using 16S-rRNA analysis.

Introduction

The wall-less eubacteria of the genera *Mycoplasma, Ureaplasma, Spiroplasma, Acholeplasma, Anaeroplasma,* and *Asteroleplasma* are grouped together in the Class *Mollicutes.* Phylogenetic analyses of 5S- and 16S-rRNA sequences have revealed that the mollicutes cluster with the low-G+C subdivision of Gram-positive eubacteria that includes the bacilli-lactobacilli-streptococci branch (Rogers et al., 1985; Woese, 1987; Woese et al., 1980). Among members of the low-G+C Gram-positive eubacteria, the mollicutes are most closely related to *Clostridium innocuum, Clostridium ramosum, Erysipelothrix rhusiopathiae, Lactobacillus catenaformis* (*Lactobacillus catenaforme*), *Lactobacillus vitulinus,* and *Streptococcus pleomorphus* (Ludwig et al., 1988; Rogers et al., 1985; Woese, 1987; Woese et al., 1980, 1985; Weisburg et al., 1989).

The mollicutes and their walled phylogenetic relatives have few phenotypic similarities. The only comparative study of a phenotype of the mollicutes and their walled phylogenetic relatives was conducted by Pellegrin et al. (1988). They found that *C. innocuum, C. ramosum,* and six species of mollicutes (belonging to four genera) were all resistant

to high levels of rifampin. Generally, eubacteria are sensitive to rifampin, an inhibitor of RNA polymerase; some mollicutes, however, are resistant (Gadeau et al., 1986).

Some members of the *Mycoplasmatales* and the *Acholeplasmatales* have activities for (deoxy)ribonucleoside kinases that are dependent on pyrophosphate (PP_i) as a cofactor (McElwain et al., 1988; Tryon and Pollack, 1984), and *Acholeplasma* spp. have PP_i-dependent phosphofructokinase (PP_i-PFK) activity (DeSantis et al., 1989; Pollack and Williams, 1986). Among the anaerobic mollicutes, both *Anaeroplasma intermedium* (McElwain et al., 1988) and *Asteroleplasma anaerobium* (Petzel et al., 1989) had activities for PP_i-nucleoside kinases. *An. intermedium* also had activity for PP_i-PFK, whereas *As. anaerobium* had activity for a third PP_i-dependent enzyme, pyruvate, orthophosphate dikinase (PPDK; Petzel et al., 1989). PP_i-nucleoside kinase has not been reported in any organisms other than some mollicutes (McElwain et al., 1988; Pollack et al., 1989; Tryon and Pollack, 1984). ATP is the phosphate donor in the PFK reaction instead of PP_i in the great majority of bacteria. PPDK, which converts phospho*enol*pyruvate (PEP) to pyruvate, has been reported in only a few prokaryotes (Wood, 1985).

Because PP_i-dependent enzymes are generally rare among prokaryotes but occur more frequently among the mollicutes, and because PP_i may be an evolutionary precursor of ATP (Kulaev and Vagabov, 1983; Wood, 1985), we examined the walled phylogenetic relatives of the mollicutes and some of the *Anaeroplasmataceae* for four PP_i-dependent enzymes: PP_i-PFK, PPDK,

PEP carboxytransphosphorylase, and PP_i-acetate kinase. We also assayed these bacteria for some analogous ATP-dependent enzyme activities.

Materials and Methods

Strains

Clostridium innocuum ATCC 14501^T, *Lactobacillus catenaformis* ATCC 25536^T (=DSM 20559^T), *Lactobacillus vitulinus* ATCC 27783^T, and *Streptococcus pleomorphus* ATCC 29734^T (=DSM 20574^T) were obtained through the American Type Culture Collection, Rockville, MD. *Anaeroplasma intermedium* 5LA and 7LA^T (=ATCC 43166^T; Robinson and Allison, 1975; Robinson and Freundt, 1987) and *Asteroleplasma anaerobium* 161^T (=ATCC 27880^T; Robinson and Freundt, 1987) were obtained from I. M. Robinson, National Animal Disease Center (NADC), U.S. Department of Agriculture, Ames, IA. *Erysipelothrix rhusiopathiae* AVR-9 was obtained from R. L. Wood, NADC. *Clostridium innocuum* 7207 and *Clostridium ramosum* 8546 were received through the Anaerobe Laboratory, Virginia Polytechnic Institute, Blacksburg.

Cultivation and extract preparation

The mollicutes were harvested from 1.2 L of S-2 broth (≤0.5% inocula, incubations of 4-6 d at 37°C) as described previously (Petzel et al., 1989). *E. rhusiopathiae* was grown (0.8% inoculum, 2 d at 37°C) in 1.2 L of S-2 broth with the following modifications: The medium was supplemented with heat-inactivated horse serum to 1.7 % (vol/vol) and MES

[2-(N-morpholino)ethanesulfonic acid] to 10m*M*; the lipids and Na_2CO_3, were deleted and the culture was incubated without anaerobic precautions. All other species were grown in 800 ml of S-2 broth without the lipids (0.25% inocula, 20-22 h at 37°C).

Extracts of the mollicutes were prepared by osmotic lysis (Petzel et al., 1989). Pellets of walled bacteria were resuspended in the same lysis buffer and were passed through a French pressure cell 2-4 times. Lysates were clarified by centrifugation at 8,800 x g_{max} at 4°C for 15 min and then again at 230,000 x g_{max} at 4° for 1 h unless noted otherwise. Aliquots of the cell-free extracts were either frozen at -75°C or dialysed against 100 volumes of lysis buffer (20 h, 4°C, one change) before being frozen. Some cell-free extracts were frozen under argon; no differences in activity were observed between these preparations and those with which no anaerobic precautions were taken. Protein concentrations were determined by using the BCA Protein Assay Reagent (Pierce, Rockford, IL), with bovine serum albumin as the standard.

Enzyme assays

Biochemicals were obtained from Sigma. All potentially labile substrates and cofactors were prepared in buffers and stored in the manner appropriate for maximum stability (Beutler and Supp, 1983). ATP was determined by the method of Trautschold et al. (1985).

ATP-dependent 6-phosphofructokinase (ATP-PFK; EC 2.7.1.11) and PP_i-dependent 6-phosphofructokinase (PP_i-PFK; EC 2.7.1.90) were assayed in the forward direction by method A of O'Brien et al. (1975). PP_i-PFK was also assayed in the reverse direction by method B (O'Brien et al., 1975). Pyruvate, orthophosphate dikinase (PPDK; EC 2.7.9.1) was assayed by determining the appearance of ATP [Ernst et al., 1986; $(NH_4)_6Mo_7O_{24}$ was omitted] and by determining the appearance of pyruvate (Evans and Wood, 1971; $MgCl_2$ was decreased to 7.4 m*M*). PEP carboxytransphosphorylase (EC 4.1.1.38) was assayed by following the appearance of oxaloacetate by a modification of the method of Wood et al. (1969). The mixture of $KHCO_3$, $MgCl_2$, and potassium phosphate buffer in a serum bottle was sparged with CO_2 for 30 min, sealed with a septum, and aliquots were withdrawn with a syringe as needed. In addition, the concentration of $CoCl_2$ was reduced to 10 μ*M*, and dithiothreitol was substituted for β-mercaptoethanol. PEP carboxytransphosphorylase was also assayed by determining the appearance of PP_i; the assay mixture used for the oxaloacetate assay was modified by deleting NADH and malate dehydrogenase and increasing $CoCl_2$ to 100 μ*M*. The reaction mixtures were incubated under CO_2 for 30 min at 37°C; mixtures without PEP served as controls. Pyrophosphate concentrations were determined with a commercial assay kit (Sigma) according to the manufacturer's directions. Control values were subtracted from those obtained by using complete assay mixtures. ATP-dependent acetate kinase (EC 2.7.2.1) reactions in the direction of acetyl phosphate formation were conducted by the method of Bowman et al. (1976), with potassium

acetate added to 50 m*M*, and sodium succinate (a positive effector in *Escherichia coli*) added to 10 m*M*. For each strain, a second assay mixture with boiled cell-free extract was used as the blank for the spectrophotometric determination of acetyl hydroxamate by the method of Skarstedt and Silverstein (1976). PP_i-dependent acetate kinase (EC 2.7.2.12) was determined similarly, except that $MgCl_2$ was reduced to 0.75 m*M* to prevent precipitation with PP_i. Alternatively, to circumvent potential inhibition by PP_i of color formation in the hydroxylamine assay for acetyl phosphate in the PP_i-acetate kinase reaction, the method of Reeves and Guthrie (1975) was used. Sodium dithionite, which reportedly stimulates this reaction in *Desulfotomaculum ruminis* (Liu and Peck, 1981), was added to 25 m*M*. Pyruvate kinase (EC 2.7.1.40) was assayed by the method of Smart and Pritchard (1979); PEP was added to 4 m*M*. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was assayed anaerobically by the method described by Petzel et al. (1989).

Results and Discussion

Woese and co-workers (Weisburg et al., 1989) used 16S-rRNA analyses to determine that the walled bacteria phylogenetically related to the mollicutes can be divided into monophyletic subgroups. *Clostridium innocuum* clustered with *Streptococcus pleomorphus*, and *Clostridium ramosum* clustered with *Lactobacillus catenaformis* and *L. vitulinus*. *Erysipelothrix rhusiopathiae* did not belong to either cluster, but Weisburg et al. (1989) reported that there were some data to suggest

that *E. rhusiopathiae* was more closely related to the *C. innocuum-S. pleomorphus* cluster than to the *C. ramosum*-lactobacilli cluster.

The purpose of the present study was twofold: (i) to determine if the walled bacteria that are related to the mollicutes possessed pyrophosphate-dependent (PP_i-dependent) enzymes as the mollicutes do, thereby providing a phenotypic indication of their phylogenetic relatedness and (ii) to determine if the distribution of PP_i-dependent enzymes among the six walled bacteria complements the phylogenetic subdivisions of this group as proposed by Weisburg et al. (1989).

Functional and structural patterns of various enzymes have been used as the bases for taxonomic and phylogenetic classifications (Byng et al., 1982; Jensen, 1985; Weitzman, 1980). Weitzman (1980) reported that regulatory and size characteristics of citrate synthase and succinate thiokinase can be used to separate Gram-positive and Gram-negative bacteria and also aerobic Gram-negative bacteria from facultative anaerobes. Jensen and co-workers (Byng et al., 1982; Jensen, 1985) have made phylogenetic studies about various groups of bacteria and their pathway for aromatic amino acid biosynthesis by studying differences in 3isozymes, cofactor specificities, allosteric controls, and alternative biochemical steps; they determined that phylogenetic clusters of bacteria share distinctive biochemical features. However, these workers stressed that any conclusions about the phylogeny or evolution of organisms or their biochemical pathways must be made within the framework of phylogenetic relationships previously determined by other established

methods, such as rRNA analysis (Jensen, 1985).

The data reported here represent enzyme activities determined *in vitro* (i.e., cell extracts); thus, extrapolations to quantitative conclusions about conditions *in vivo* would be tenuous. When we could not detect an enzyme activity, where possible, enzyme from a commercial source or a known-positive organism was added to the putatively inactive cell-free extract to determine the efficacy of the assay system. Previous studies have demonstrated that, except for well-documented isolated cases (Abbe et al., 1982), most enzymes of carbohydrate metabolism (including PP_i-dependent enzymes) found in both aerobic and anaerobic bacteria are not O_2-labile (Cruden et al., 1983; Joyner and Baldwin, 1966; Petzel et al., 1989; Roberton and Glucina, 1982).

Phosphofructokinase

Among walled bacteria, PP_i-PFK has previously been detected in a *Clostridium* sp. isolated from the cockroach gut (Cruden et al., 1983), *Propionibacterium freundenreichii* and *P. acidipropionici* ("*P. arabinosum*") (O'Brien et al., 1975; Wood, 1985; Wood and Goss, 1985), some *Bacteroides* spp. (Roberton and Glucina, 1982), and *Deleya* spp. (Sawyer et al., 1977). Among the *Mollicutes*, PP_i-PFK has been detected in *Acholeplasma* spp., but the PFK activities among *Mycoplasma* spp. and *Spiroplasma* spp., when present, are active with ATP but not PP_i (DeSantis et al., 1989; Lake-Bullock et al., 1989; Pollack and Williams, 1986; Pollack et al., 1989). In the anaerobic mollicutes, *Anaeroplasma*

intermedium 5LA has PP_i-PFK activity and *Asteroleplasma anaerobium* 161^T has ATP-PFK activity (Petzel et al., 1989).

In the present study, *C. innocuum* and *S. pleomorphus* (one of the clusters within the group of six) and *E. rhusiopathiae* had PP_i-PFK activities in both the forward and reverse directions. However, *C. ramosum*, *L. catenaformis*, and *L. vitulinus* (another cluster) had only ATP-PFK activity (Table 1).

Parenthetically, PFK activities were sometimes detected in the two strains of *C. innocuum*, in *S. pleomorphus*, and in the two strains of *An. intermedium* when ATP was substituted for PP_i; this was not observed with *E. rhusiopathiae*. This ATP-linked PFK activity was not detected in all extract preparations of these organisms. Indeed, in a previous study, we did not detect ATP-linked activity in *An. intermedium* 5LA (Petzel et al., 1989). In the present study, the ATP-linked activities were always less than those of the PP_i-PFK activities in these species (Table 1). Among the other microorganisms that have PP_i-PFK, the *Clostridium* sp. (Cruden et al., 1983), *Euglena gracilis* (Miyatake et al., 1984), *Propionibacterium* spp. (O'Brien et al., 1975; Wood and Goss, 1985), and the *Bacteroides* spp. (Roberton and Glucina, 1982) have been reported to also have ATP-PFK, but *Deleya* spp. had only PP_i-PFK (Sawyer et al., 1977). Reeves and co-workers (Reeves, 1987) initially reported that *Entamoeba histolytica* had both ATP- and PP_i-PFK activities. Subsequent investigations revealed that the seeming ATP-PFK activity resulted from the generation of PP_i from ATP by the glycogen-forming apparatus; strong

centrifugal forces removed the ATP-linked activity from the extracts (Reeves, 1987). Roberton and Glucina (1982) reported that less activity was detected in extracts of *Bacteroides* spp. subjected to a centrifugal force of 190,000 x *g* (1 h) than in extracts subjected to a centrifugal force of 27,000 x *g* (20 min). The ATP-PFK activity of *P. freundenreichii* was stable to a centrifugal force of 124,000 x *g* (1 h) (O'Brien et al., 1975). In the present study, we routinely used a centrifugal force of 230,000 x g_{max} (1 h). We did not observe any marked reduction in ATP-linked activity in lysates of *An. intermedium* 5LA clarified by a centrifugal force of 330,000 x g_{max} (120 min) compared with lysates clarified at 27,000 x g_{max} (20 min) (data not shown).

To ensure that this ostensive ATP-PFK activity was not caused by contamination of assay components with PP_i (Reeves, 1987), we performed the following investigations. Using a PP_i assay kit (Sigma), we did not detect PP_i in either the ATP stock solutions or in cell-free extracts of *An. intermedium* 5LA. PP_i-PFK purified from *P. freundenreichii* (Sigma) was not active with our ATP solutions. To determine if extracts of *An. intermedium* 5LA could generate PP_i from ATP, we assayed for the appearance of PP_i in ATP-PFK reaction mixtures containing all components except fructose 6-phosphate; no PP_i was detected. (Conversely, extracts of *An. intermedium* 5LA and *C. innocuum* 7207 did not generate detectable amounts of ATP from PP_i.) Furthermore, the inclusion of 5 units of yeast inorganic pyrophosphorylase per ml of PFK assay mix diminished PP_i-dependent activities, but not ATP-dependent activities, in

An. intermedium 5LA and *C. innocuum* 7207 (data not shown). These observations suggest the presence of *bona fide* ATP-PFK in *C. innocuum, S. pleomorphus,* and *An. intermedium;* purification of the ATP-dependent activity from the PP_i-dependent activity would provide definite proof. The observations of the putative ATP-PFK aside, the occurrence of PP_i-PFK among some of the mollicutes and three of their six walled relatives is indicative of their phylogenetic relatedness.

The occurrence of PP_i-PFK in *Acholeplasma* spp. (DeSantis et al., 1989; Pollack and Williams, 1986) and in *An. intermedium* (Petzel et al., 1989; this study) and the absence of PP_i-PFK in other mollicutes (including *As. anaerobium* 161^T) (DeSantis et al., 1989; Petzel et al., 1989) correlate with the subgroups of mollicutes determined by Weisburg et al. (1989) using 16S-rRNA sequence analyses. They reported that acholeplasmas grouped with *Anaeroplasma* spp., to the exclusion of the other mollicutes.

Pyruvate, orthophosphate dikinase and pyruvate kinase

PPDK converts PEP, AMP, and PP_i to pyruvate, ATP, and P_i. This enzyme has previously been reported in *P. freundenreichii, P. acidipropionici, Clostridium symbiosum* ("*Bacteroides symbiosus*"), some *Acetobacter* spp., the clostridium isolated from the cockroach gut, and three anoxygenic photosynthetic bacteria (Cruden et al., 1983; Ernst et al., 1986; Evans and Wood, 1971; Wood, 1985; Wood and Goss, 1985). Among the mollicutes, PPDK has been detected in *As. anaerobium* (Petzel et al.,

1989) but not in any of the ten other mollicutes examined (Manolukas et al., 1988).

In the present study, PPDK activity was detected in *C. innocuum, C. ramosum, L. catenaformis, L. vitulinus, S. pleomorphus, and As. anaerobium*, but not in *E. rhusiopathiae* or *An. intermedium* (Table 2). The activities were detected by assaying both for the conversion of PEP to pyruvate and the conversion of AMP to ATP. The fact that *As. anaerobium* is the only mollicute reported to possess PPDK is phenotypic evidence supportive of the unique phylogenetic status of this organism among the mollicutes (Weisburg et al., 1989). No conclusions about the specific relationship (if indeed any exists) between *As. anaerobium* and the walled relatives can be made on the basis of the present information concerning PPDK.

All the walled relatives of the mollicutes except *E. rhusiopathiae* had pyruvate kinase activity, a second enzyme that interconverts PEP and pyruvate; the activity for *S. pleomorphus* was low and variable (Table 3). In previous studies, the pyruvate kinases of *Mycoplasma* spp., *Acholeplasma* spp., *Ureaplasma urealyticum*, and *An. intermedium* 5LA (Cocks et al., 1985; Manolukas et al., 1988; Petzel et al., 1989) were detected in the absence of allosteric activators. In contrast, the pyruvate kinase activities in four of five walled bacteria determined in the present study to possess the enzyme were significantly increased by one or more activators (Table 3). Fructose 1,6-bisphosphate (Fru-1,6-P_2) had only moderate or no effects on the pyruvate kinase activities of

C. innocuum, *S. pleomorphus*, and *C. ramosum*; AMP and glucose 6-phosphate (Glc-6-*P*) were more effective.

Fru-1,6-P_2 activates pyruvate kinases in a wide variety of bacteria (Sanwal, 1970; Smart and Pritchard, 1979; Yamada and Carlsson, 1975); fewer bacteria have pyruvate kinases activated by Glc-6-*P* but not Fru-1,6-P_2 (Smart and Pritchard, 1979; Yamada and Carlsson, 1975). Yamada and Carlsson (1975) suggested that organisms that have both the Embden-Meyerhof-Parnas and pentose phosphate pathways for the degradation of glucose may use Fru-1,6-P_2 as an activator for pyruvate kinase because Glc-6-*P* is the branch point between the two pathways. However, organisms that lack the oxidative portion of the pentose phosphate pathway may use Glc-6-*P* as the primary activator for pyruvate kinase. We did not detect Glc-6-*P* dehydrogenase (the first enzyme in the oxidative portion of the pentose phosphate pathway) in cell-free extract preparations of any of the bacteria except for *S. pleomorphus* (4.9 nmol/min/mg).

In the two lactobacilli, AMP was the best activator of pyruvate kinase; indeed, no activity was observed in extracts of *L. catenaformis* in the absence of AMP (Table 3). Thus, the two lactobacilli differed from the third member of this phylogenetic subgroup, *C. ramosum*. Weisburg et al. (1989) reported that although the lactobacilli cluster with *C. ramosum*, they are more closely related to each other than to *C. ramosum*.

The pyruvate kinase activity of *An. intermedium* was not significantly increased by any of the three effectors (Table 3). If the

absence of allosteric activation of pyruvate kinase is confirmed for mollicutes in general, this would indicate that allosteric regulation of this enzyme arose in the walled relatives after the wall-less organism(s) diverged from the walled progenitor(s). The absence of pyruvate kinase activity in *As. anaerobium*, an enzyme detected in all other mollicute species examined, is further phenotypic evidence of its distinct phenotypic status among the *Mollicutes*.

We could not detect activity for either PPDK or pyruvate kinase in extracts of *E. rhusiopathiae* (Tables 2, 3). The addition of Glc-6-*P*, Fru-1,6-P_2, or AMP had no effect. Robertson and McCullough (1968) reported that *E. rhusiopathiae* degrades glucose via the Embden-Meyerhof-Parnas pathway, based on the distribution of ^{14}C among products of [^{14}C]glucose degradation. In the present study, the absence of detectable PPDK or pyruvate kinase activities in *E. rhusiopathiae* AVR-9 indicates either that our methods were not suitable for detecting these enzymes in this organism, or that the avirulent vaccine strain used in the present study differed from strain S-192, which was used by Robertson and McCullough (1968).

PEP Carboxytransphosphorylase

PEP carboxytransphosphorylase occurs in *P. freundenreichii*, *P. acidipropionici*, some *Rhodopseudomonas* spp., *Brevibacterium ammoniagenes* ("*Corynebacterium ammoniagenes*"), and the clostridium isolated from the cockroach gut (Cruden et al., 1983; Wood, 1985; Wood and Goss, 1985; Wood

et al., 1969). *Acholeplasma laidlawii* was reported to possess activity for PEP carboxytransphosphorylase (Beaman and Pollack, 1984; Tryon and Pollack, 1984), but more recently, workers from the same laboratory have not been able to confirm this activity (Manolukas et al., 1988). In the present study, activities for PEP carboxytransphosphorylase were detected by two methods in *C. innocuum*, *S. pleomorphus*, and *C. ramosum* (Table 4). Low levels of the enzyme were detected in the lactobacilli and the two strains of *An. intermedium* by the method detecting PP_i, but not by the method detecting oxaloacetate (Table 4). No PEP carboxytransphosphorylase activity was detected in *E. rhusiopathiae* or *As. anaerobium* (Table 4). Thus, PEP carboxytransphosphorylase is an additional PP_i-dependent enzyme possessed by some of the walled relatives of the *Mollicutes*.

Acetate kinase

PP_i-acetate kinase has been reported in *Entamoeba histolytica* (Reeves and Guthrie, 1975) and in Gram-negative sulfate-reducing anaerobes (Liu and Peck, 1981; but see Wood, 1985). We did not detect PP_i-acetate kinase activity in the direction of acetyl phosphate in any of the organisms examined; duplicate extracts of all organisms were tested by the modified method of Bowman et al. (1976), and single extracts of *C. innocuum* ATCC 14501^T, *C. ramosum*, *E. rhusiopathiae*, *L. catenaformis*, and *S. pleomorphus* were tested by the method of Reeves and Guthrie (1975). However, all species had activity for ATP-acetate kinase

in the direction of acetyl phosphate [means of determinations (μmol/min/mg) from two batches of cells except as noted]: *C. innocuum*, strain ATCC 14501^T, 132; strain 7207, 355; *S. pleomorphus*, 228; *C. ramosum*, 77.9; *L. catenaformis*, 27.2; *L. vitulinus*, 16.4; *E. rhusiopathiae*, 155; *An. anaerobium* strain 5LA, 30.3; strain 7LA^T (single batch), 10.2; and *As. anaerobium* (single batch), 424. [We did not attempt to determine ATP-acetate kinase activity in the direction of acetate and ATP synthesis because of the high levels of adenylate kinase activity in extracts of *An. intermedium* 5LA and *As. anaerobium* 161^T. Adenylate kinase, which converts two moles of ADP to one mole of ATP and one mole of AMP, interfered with the assay for ATP production in the ATP-acetate kinase reaction.] Muhlrad et al. (1981) did not detect ATP-acetate kinase activity in *Anaeroplasma abactoclasticum* 6-1^T or *An. intermedium* 7LA^T. These researchers used the hydroxylamine method to detect acetyl phosphate production (Muhlrad et al., 1981; Skarstedt and Silverstein, 1976), the same method employed in the present study (Bowman et al., 1976; Skarstedt and Silverstein, 1976). Kotzé (1968) reported that the hydroxylamine method is subject to interference by substances in the sample and that it does not give reproducible results, but other researchers have used this method in extensive kinetic analyses of acetate kinase from *Veillonella alcalescens* (Bowman et al., 1976) and *E. coli* (Skarstedt and Silverstein, 1976). Robinson and co-workers (Robinson and Allison, 1975; Robinson et al., 1975) reported that acetate is produced from starch by *An. abactoclasticum* 6-1^T and *An. intermedium*

strains 7LA^T and 5LA. Thus, the correlation between the production of acetate from starch and the production of acetyl phosphate from acetate and ATP suggests that these strains do possess acetate kinase.

Phylogenetic implications

The possession of a PP_i-dependent enzyme(s) by any two given species does not, by itself, necessarily denote a phylogenetic relationship between those species. For example, the bacteria examined in the present study and *Propionibacterium* spp. share several PP_i-dependent enzymes in common, but the propionibacteria belong to a different subdivision of Gram-positive eubacteria, as determined by rRNA analysis (Woese, 1987). However, given that the bacteria examined in the present study are phylogenetically related to the mollicutes, the occurrence of one or more PP_i-dependent enzymes in each of these bacteria (enzymes that occur in other organisms infrequently) is phenotypic evidence that supports the proposed phylogenetic relationship between the mollicutes and their walled relatives. Two unrelated clostridia, *Clostridium bifermentans* and *C. butyricum*, did not have detectable PP_i-PFK or PPDK activities (data not shown).

Among mollicutes, four PP_i-dependent enzymes have been reported: PP_i-PFK, PPDK, PEP carboxytransphosphorylase, and PP_i nucleoside kinase. PP_i nucleoside kinase has been found in all three orders of the Class *Mollicutes* (McElwain et al., 1988; Pollack et al., 1989), whereas the other three enzymes have been reported only among members of

the *Acholeplasmatales* and the *Anaeroplasmatales* (DeSantis et al., 1989; Manolukas et al., 1988; Petzel et al., 1989; this study). The occurrence of multiple PP_i-dependent enzymes among the *Acholeplasmatales*, the *Anaeroplasmatales*, and the walled phylogenetic relatives and the reported lack of any PP_i-dependent enzymes besides PP_i nucleoside kinase among the *Mycoplasmatales*, support the rRNA-sequence analyses that suggest the acholeplasmas and anaeroplasmas represent the first branch(es) from the walled group (Rogers et al., 1985; Woese, 1987).

The findings reported here are phenotypic indicators of the phylogenetic relatedness of the mollicutes to certain walled bacteria. Distributions of these enzymes substantiate the subdivision of this phylogenetic group by 16S-rRNA analyses. Characterization of these enzymes at the structural level (Selander et al., 1986) could help elucidate evolutionary lineages within the *Mollicutes* and their walled relatives.

Table 1. 6-Phosphofructokinase (PFK) activities in walled bacteria phylogenetically related to *Mollicutes* and in strictly anaerobic mollicutes

Species[a]	PFK activity (nmol/min/mg)[b]		
	PP_i-PFK		ATP-PFK
	Forward reaction	Reverse reaction	
Clostridium innocuum ATCC 14501^T	607	620	0.41[c]
Clostridium innocuum 7207	255	338	13.6[c]
Streptococcus pleomorphus ATCC 29734^T	646	743	14.4[c]
Clostridium ramosum 8546	NA[d]	NA	67.8
Lactobacillus catenaformis ATCC 25536^T	NA	NA	203
Lactobacillus vitulinus ATCC 27783^T	NA	NA	53.2

[a]Species are grouped by phylogenetic subdivisions as determined by Weisburg et al. (1989).

[b]Data are means of determinations from two batches of cells, except *An. intermedium* 7LA^T and *As. anaerobium* 161^T (single batches of these were tested; see also footnote c). See text for details of assays.

[c]ATP-linked PFK activities in these organisms were detected in only one extract preparation (except for *An. intermedium* 5LA; the activity was detected in four preparations of this strain). See text for discussion.

[d]NA, no activity detected.

Table 1 (Continued)

Species[a]	PFK activity (nmol/min/mg)[b]		
	PP_i-PFK		ATP-PFK
	Forward reaction	Reverse reaction	
Erysipelothrix rhusiopathiae AVR-9	924	957	NA
Anaeroplasma intermedium 5LA	378	191	38.7[c]
Anaeroplasma intermedium 7LA[T]	119	80.2	4.2[c]
Asteroleplasma anaerobium 161[T]	NA	NA	37.4

TABLE 2. Pyruvate, orthophosphate dikinase (PPDK) activities in walled bacteria phylogenetically related to *Mollicutes* and in strictly anaerobic mollicutes

Species[a]	PPDK activity (nmol/min/mg)[b]	
	Assay for ATP	Assay for pyruvate
Clostridium innocuum ATCC 14501^T	9.7	11.7
Clostridium innocuum 7207	13.4	12.1
Streptococcus pleomorphus ATCC 29734^T	30.0	29.6
Clostridium ramosum 8546	67.1	94.4
Lactobacillus catenaformis ATCC 25536^T	16.9	105
Lactobacillus vitulinus ATCC 27783^T	23.1	30.5
Erysipelothrix rhusiopathiae AVR-9	NA[c]	NA
Anaeroplasma intermedium 5LA	NA	NA
Anaeroplasma intermedium 7LA^T	NA	NA
Asteroleplasma anaerobium 161^T	112	335

[a]Species are grouped by phylogenetic subdivisions as determined by Weisburg et al. (1989).

[b]Data are the means of determinations from two batches of cells (except *An. intermedium* 7LA^T and *As. anaerobium* 161^T; single batches of these were tested). See text for details of assays.

[c]NA, no activity detected.

TABLE 3. Effects of three potential allosteric activators on pyruvate kinase activities in walled bacteria phylogenetically related to *Mollicutes* and in strictly anaerobic mollicutes

Species[a]	Pyruvate kinase activity (nmol/min/mg) with[b]:			
	No additives	0.5-m*M* Fru-1,6-P_2	1.5-m*M* AMP	0.5m-*M* Glc-6-*P*
Clostridium innocuum ATCC 14501^T	7.0	10	49	126
Clostridium innocuum 7207	14	31	43	280
Streptococcus pleomorphus ATCC^T	1.3	0.52	NA	4.4
Clostridium ramosum 8546	20	19	196	539
Lactobacillus catenaformis ATCC^T	NA	NA	257	NA
Lactobacillus vitulinus ATCC^T	124	316	813	660
Erysipelothrix rhusiopathiae AVR-9	NA	NA	NA	NA

[a]Species are grouped by phylogenetic subdivisions as determined by Weisburg et al. (1989).

[b]Data are the means of determinations from two batches of cells (except *An. intermedium* 7LA^T and *As. anaerobium* 161^T; single batches of these were tested). See text for details of assays. Fru-1,6-P_2, fructose 1,6-bisphosphate; AMP, adenosine 5'-monophosphate; Glc-6-*P*, glucose 6-phosphate; NA, no activity detected.

Table 3 (Continued)

Species[a]	Pyruvate kinase activity (nmol/min/mg) with[b]:			
	No additives	0.5-m*M* Fru-1,6-P_2	1.5-m*M* AMP	0.5m-*M* Glc-6-*P*
Anaeroplasma intermedium 5LA	168	159	171	175
Anaeroplasma intermedium 7LA^T	81	85	90	80
Asteroleplasma anaerobium 161^T	NA	NA	NA	NA

TABLE 4. PEP carboxytransphosphorylase activities in walled bacteria phylogenetically related to *Mollicutes* and in strictly anaerobic mollicutes

Species[a]	PEP carboxytransphosphorylase activity (nmol/min/mg)[b]	
	Assay for oxaloacetate	Assay for PP_i
Clostridium innoccum ATCC 14501^T	5.5	22.1
Clostridium innocuum 7207	21.6	80.5
Streptococcus pleomorphus ATCC 29734^T	27.1	25.3
Clostridium ramosum 8546	8.2	5.5
Lactobacillus catenformis ATCC^T	NA[c]	2.5
Lactobacillus vitulinus ATCC 27783^T	NA	0.71
Erysipelothrix rhusiopathiae AVR-9	NA	NA
Anaeroplasma intermedium 5LA	NA	7.4
Anaeroplasma intermedium 7LA^T	NA	5.7
Asteroleplasma anaerobium 161^T	NA	NA

[a]Species are grouped by phylogenetic subdivisions as determined by Weisburg et al. (1989).

[b]Data are the means of determinations from two batches of cells (except *An. intermedium* 7LA^T and *As. anaerobium* 161^T; single batches of these were assayed twice). See text for details of assays.

[c]NA, no activity detected.

References Cited

Abbe, K., S. Takahashi, and T. Yamada. 1982. Involvement of oxygen-sensitive pyruvate formate-lyase in mixed-acid fermentation by *Streptococcus mutans* under strictly anaerobic conditions. J. Bacteriol. 152:175-182.

Beaman, K. D., and J. D. Pollack. 1984. Enzymatic assimilation of ^{14}C from $NaH^{14}CO_3$ by extracts of *Acholeplasma laidlawii* B-PG9. Yale J. Biol. Med. 57:897 (Abstr.).

Beutler, H.-O., and M. Supp. 1983. Coenzymes, metabolites, and other biochemical reagents. Pages 328-393 *in* H. U. Bergmeyer, J. Bergmeyer, and M. Graßl (eds.), Methods of enzymatic analysis. Third edition. Volume II. Samples, reagents, assessment of results. Verlag Chemie, Deerfield Beach, FL.

Bowman, C. M., R. O. Valdez, and J. S. Nishimura. 1976. Acetate kinase from *Veillonella alcalescens*. J. Biol. Chem. 251:3117-3121.

Byng, G. S., J. F. Kane, and R. A. Jensen. 1982. Diversity in the routing and regulation of complex biochemical pathways as indicators of microbial relatedness. Crit. Rev. Microbiol. 9:227-252.

Cocks, B. G., F. A. Brake, A. Mitchell, and L. R. Finch. 1985. Enzymes of intermediary carbohydrate metabolism in *Ureaplasma urealyticum* and *Mycoplasma mycoides* subsp. *mycoides*. J. Gen. Microbiol. 131:2129-2135.

Cruden, D. L., W. E. Durbin, and A. J. Markovetz. 1983. Utilization of PP_i as an energy source by a *Clostridium* sp. Appl. Environ. Microbiol. 46:1403-1408.

DeSantis, D., V. V. Tryon, and J. D. Pollack. 1989. Metabolism of mollicutes: The Embden-Meyerhof-Parnas pathway and the hexose monophosphate shunt. J. Gen. Microbiol. 135:683-691.

Ernst, S. M., R. J. A. Budde, and R. Chollet. 1986. Partial purification and characterization of pyruvate,orthophosphate (sic) dikinase from *Rhodospirillum rubrum*. J. Bacteriol. 165:483-488.

Evans, H. J., and H. G. Wood. 1971. Purification and properties of pyruvate phosphate dikinase from propionic acid bacteria. Biochemistry 10:721-729.

Gadeau, A.-P., C. Mouches, and J. M. Bove. 1986. Probable insensitivity of mollicutes to rifampin and characterization of spiroplasmal DNA-dependent RNA polymerase. J. Bacteriol. 166:824-828.

Jensen, R. A. 1985. Biochemical pathways in prokaryotes can be traced backward through evolutionary time. Mol. Biol. Evol. 2:92-108.

Joyner, A. E., Jr., and R. L. Baldwin. 1966. Enzymatic studies of pure cultures of rumen microorganisms. J. Bacteriol. 92:1321-1330.

Kotzé, J. P. 1968. An enzymatic optical method for the determination of nanomole quantities of acetyl phosphate. J. S. Afr. Chem. Inst. 21:105-112.

Kulaev, I. S., and V. M. Vagabov. 1983. Polyphosphate metabolism in micro-organisms. Adv. Microb. Physiol. 24:83-171.

Lake-Bullock, M. H. V., W. T. Blevins, S. K. McGlynn, and T. L. Rhoads. 1989. Pathways of glucose catabolism in *Spiroplasma*. Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, G-39, p. 155.

Liu, C.-L., and H. D. Peck, Jr. 1981. Comparative bioenergetics of sulfate reduction in *Desulfovibrio* and *Desulfotomaculum* spp. J. Bacteriol. 145:966-973.

Ludwig, W., M. Weizenegger, R. Kilpper-Bälz, and K. H. Schleifer. 1988. Phylogenetic relationships of anaerobic streptococci. Int. J. Syst. Bacteriol. 38:15-18.

Manolukas, J. T., M. F. Barile, D. K. F. Chandler, and J. D. Pollack. 1988. Presence of anaplerotic reactions and transamination, and the absence of the tricarboxylic acid cycle in mollicutes. J. Gen. Microbiol. 134:791-800.

McElwain, M. C., D. K. F. Chandler, M. F. Barile, T. F. Young, V. V. Tryon, J. W. Davis, Jr., J. P. Petzel, C.-J. Chang, M. V. Williams, and J. D. Pollack. 1988. Purine and pyrimidine metabolism in *Mollicutes* species. Int. J. Syst. Bacteriol. 38:417-423.

Miyatake, K., T. Enomoto, and S. Kitaoka. 1984. Detection and subcellular distribution of pyrophosphate: D-Fructose 6-phosphate phosphotransferase (PFP) in *Euglena gracilis*. Agric. Biol. Chem. 48:2857-2859.

Muhlrad, A., I. Peleg, J. A. Robertson, I. M. Robinson, and I. Kahane. 1981. Acetate kinase activity in mycoplasmas. J. Bacteriol. 147:271-273.

O'Brien, W. E., S. Bowien, and H. G. Wood. 1975. Isolation and characterization of a pyrophosphate-dependent phosphofructokinase from *Propionibacterium shermanii*. J. Biol. Chem. 250:8690-8695.

Pellegrin, J. L, J. Maugein, M. Clerc, B. Leng, and C. Bebear. 1988. Activity of rifampin against mollicutes, clostridia and L forms. Abstr. Seventh Congress Int. Organ. Mycoplasmol. 1988, abstr. P 114.

Petzel, J. P., M. C. McElwain, D. DeSantis, J. Manolukas, M. V. Williams, P. A. Hartman, M. J. Allison, and J. D. Pollack. 1989. Enzymic activities of carbohydrate, purine, and pyrimidine metabolism in the *Anaeroplasmataceae* (Class *Mollicutes*). Arch. Microbiol., in press.

Pollack, J. D., and M. V. Williams. 1986. PP_i-dependent phosphofructotransferase (phosphofructokinase) activity in the mollicutes (mycoplasma) *Acholeplasma laidlawii*. J. Bacteriol. 165:53-60.

Pollack, J. D., M. C. McElwain, D. DeSantis, J. Manolukas, J. G. Tully, C.-J. Chang, R. A. Whitcomb, K. J. Hackett, and M. V. Williams. 1989. The metabolism of spiroplasmas. Int. J. Syst. Bacteriol., submitted for publication. (See also Abstr. Seventh Congress Int. Organ. Mycoplasmol. 1988, abstr. 19).

Reeves, R. E. 1987. Metabolic energy supplied by PP_i. Pages 255-259 *in* A. Torriani-Gorini, F. G. Rothman, S. Silver, A. Wright, and E. Yagil (eds.), Phosphate metabolism and cellular regulation in microorganisms. American Society for Microbiology, Washington, D.C.

Reeves, R. E., and J. D. Guthrie. 1975. Acetate kinase (pyrophosphate). A fourth pyrophosphate-dependent kinase from *Entamoeba histolytica*. Biochem. Biophys. Res. Commun. 66:1389-1395.

Roberton, A. M., and P. G. Glucina. 1982. Fructose 6-phosphate phosphorylation in *Bacteroides* species. J. Bacteriol. 150:1056-1060.

Robertson, D. C., and W. G. McCullough. 1968. Glucose catabolism of *Erysipelothrix rhusiopathiae*. J. Bacteriol. 95:2112-2116.

Robinson, I. M., and M. J. Allison. 1975. Transfer of *Acholeplasma bactoclasticum* Robinson and Hungate to the genus *Anaeroplasma* (*Anaeroplasma bactoclasticum* [Robinson and Hungate] comb.nov.): Emended description of the species. Int. J. Syst. Bacteriol. 25:182-186.

Robinson, I. M., and E. A. Freundt. 1987. Proposal for an amended classification of anaerobic mollicutes. Int. J. Syst. Bacteriol. 37:78-81.

Robinson, I. M., M. J. Allison, and P. A. Hartman. 1975. *Anaeroplasma abactoclasticum* gen.nov., sp.nov.: An obligately anaerobic mycoplasma from the rumen. Int. J. Syst. Bacteriol. 25:173-181.

Rogers, M. J., J. Simmons, R. T. Walker, W. G. Weisburg, C. R. Woese, R. S. Tanner, I. M. Robinson, D. A. Stahl, G. Olsen, R. H. Leach, and J. Maniloff. 1985. Construction of the mycoplasma evolutionary tree from 5S rRNA sequence data. Proc. Natl. Acad. Sci. USA 82:1160-1164.

Sanwal, B. D. 1970. Allosteric controls of amphibolic pathways in bacteria. Bacteriol. Rev. 34:20-39.

Sawyer, M. H., P. Baumann, and L. Baumann. 1977. Pathways of D-fructose and D-glucose catabolism in marine species of *Alcaligenes, Pseudomonas marina,* and *Alteromonas communis*. Arch. Microbiol. 112:169-172. [For current classification, see Int. J. Syst. Bacteriol. 33:793-802].

Selander, R. K., D. A. Caugant, H. Ochman, J. M. Musser, M. N. Gilmour, and T. S. Whittam. 1986. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl. Environ. Microbiol. 51:873-884.

Skarstedt, M. T., and E. Silverstein. 1976. *Escherichia coli* acetate kinase mechanism studied by net initial rate, equilibrium, and independent isotopic exchange kinetics. J. Biol. Chem. 251:6775-6783.

Smart, J. B., and G. G. Pritchard. 1979. Regulation of pyruvate kinase from *Propionibacterium shermanii*. Arch. Microbiol. 122:281-288.

Trautschold, I., W. Lamprecht, and G. Schweitzer. 1985. Adenosine 5'-triphosphate, UV-method with hexokinase and glucose-6-phosphate dehydrogenase. Pages 346-357 *in* H. U. Bergmeyer, J. Bergmeyer, and M. Graßl (eds.), Methods of enzymatic analysis. Third edition. Volume VII. Metabolites 2. VCH Publishers, Deerfield Beach, FL.

Tryon, V. V., and D. Pollack. 1984. Purine metabolism in *Acholeplasma laidlawii* B: Novel PP_i-dependent nucleoside kinase activity. J. Bacteriol. 159:265-270.

Weisburg, W. G., J. G. Tully, D. L. Rose, J. P. Petzel, H. Oyaizu, D. Yang, L. Mandelco, J. Sechrest, T. G. Lawrence, J. van Etten, J. Maniloff, and C. R. Woese. 1989. A phylogenetic analysis of the mycoplasmas: Basis for their classification. J. Bacteriol., submitted for publication.

Weitzman, P. D. J. 1980. Citrate synthase and succinate thiokinase in classification and identification. Pages 107-125 *in* M. Goodfellow and R. G. Board (eds.), Microbiological classification and identification, the Society for Applied Bacteriology Symposium Series No. 8. Academic Press, Inc., New York.

Woese, C. R. 1987. Bacterial evolution. Microbiol. Rev. 51:221-271.

Woese, C. R., J. Maniloff, and L. B. Zablen. 1980. Phylogenetic analysis of the mycoplasmas. Proc. Natl. Acad. Sci. USA 77:494-498.

Woese, C. R., E. Stackebrandt, W. Ludwig. 1985. What are mycoplasmas: The relationship of tempo and mode in bacterial evolution. J. Mol. Evol. 21:305-316.

Wood, H. G. 1985. Inorganic pyrophosphate and polyphosphates as sources of energy. Curr. Top. Cell. Regul. 26:355-369.

Wood, H. G., and N. H. Goss. 1985. Phosphorylation enzymes of the propionic acid bacteria and the roles of ATP, inorganic pyrophosphate, and polyphosphates. Proc. Natl. Acad. Sci. USA 82:312-315.

Wood, H. G., J. J. Davis, and J. M. Willard. 1969. Phosphoenolpyruvate carboxytransphosphorylase from *Propionibacterium shermanii*. Methods Enzymol. 13:297-309.

Yamada, T., and J. Carlsson. 1975. Glucose-6-phosphate-dependent pyruvate kinase in *Streptococcus mutans*. J. Bacteriol. 124:562-563.

SUMMARY AND DISCUSSION

Enzymic Activities of Strictly Anaerobic Mollicutes

The first part of my dissertation research involved the examination of two strictly anaerobic mollicutes, *Anaeroplasma intermedium* (sterol-requiring) and *Asteroleplasma anaerobium* (sterol-nonrequiring), for intracellular enzymic activities of carbohydrate metabolism. *As. anaerobium* was also examined for enzymes of purine and pyrimidine salvage and interconversion. The second part of my research involved several pyrophosphate-dependent (PP_i-dependent) enzymes. These enzymes are uncommon in prokaryotes (Wood, 1985), but they occur more frequently among mollicutes (DeSantis et al., 1989; McElwain et al., 1988; Pollack et al., 1989). Therefore, the walled bacteria that have been proposed to be phylogenetically related to the mollicutes by rRNA analyses were examined in the present study for several PP_i-dependent enzymes. In addition, the strictly anaerobic mollicutes were examined for growth stimulation by carbohydrates and for their ability to degrade colorimetric substrates for a variety of glycosidases, esterases, lipases, and peptidases (see appendices).

The first part of this dissertation determined that the pathways of carbohydrate and purine and pyrimidine metabolism in the strictly anaerobic mollicutes were similar to those reported for the facultatively anaerobic mollicutes. However, some important differences were also detected.

Other investigators have reported that the facultatively anaerobic, sterol-requiring mollicutes (the *Mycoplasmatales*) and the facultatively anaerobic, sterol-nonrequiring mollicutes (the *Acholeplasmatales*) can be distinguished by several enzymic characteristics. First, unlike the *Mycoplasmatales,* the acholeplasmas have enzymes of the oxidative shunt portion of the pentose phosphate pathway, Glc-6-*P* and 6-PG dehydrogenase (DeSantis et al., 1989; Lake-Bullock et al., 1989; O'Brien et al., 1981; Pollack, 1986; Pollack et al., 1989). Second, the acholeplasmas have PP_i-dependent phosphofructokinase (PP_i-PFK), whereas the *Mycoplasmatales* have ATP-PFK (Cocks et al., 1985; DeSantis et al., 1989; Lake-Bullock et al., 1989; Pollack et al., 1989). Third, acholeplasmas have hexokinase activity, whereas most of the *Mycoplasmatales* do not (Castrejon-Diez et al., 1963; Cocks et al., 1985; DeSantis et al., 1989; Lake-Bullock et al., 1989; Lanham et al., 1980; Pollack, 1986; Salih et al., 1983).

These three criteria that distinguish the sterol-requiring and sterol-nonrequiring, facultatively anaerobic mollicutes were not found in the present study to apply to the strictly anaerobic mollicutes. Neither *As. anaerobium* nor *An. intermedium* possessed Glc-6-*P* or 6-PG dehydrogenases. The sterol-requiring species *An. intermedium* and *An. bactoclasticum* had PP_i-PFK whereas the sterol-nonrequiring *As. anaerobium* had only ATP-PFK. Thus, the correlation between sterol requirement and cofactor specificity for PFK activity in the strictly anaerobic mollicutes is the converse of that in the facultatively anaerobic mollicutes. In regard to the third criterion, hexokinase activity,

An. intermedium possessed this enzyme but *As. anaerobium* did not. Once again, the correlation between sterol-requirement and the presence of this enzyme in the strictly anaerobic mollicutes was the converse of that in the facultatively anaerobic mollicutes.

The strictly anaerobic mollicutes were found in the present study to possess some enzymic activities that set them apart from the rest of the mollicutes. *As. anaerobium* possessed the PP_i-dependent enzyme pyruvate, orthophosphate dikinase (PPDK) that interconverts phospho*enol*pyruvate (PEP) and pyruvate. Manolukas et al. (1988) did not detect PPDK in the ten mollicutes that they studied. Instead, all the other mollicutes examined possess the ATP-linked pyruvate kinase that catalyzes an analogous reaction (Cocks et al., 1985; Davis et al., 1988, 1989; Lake-Bullock et al., 1989; Lanham et al., 1980; Manolukas et al., 1988). In the present study, *An. intermedium* was like the majority of mollicutes in that it possessed pyruvate kinase but not PPDK activity.

Except for malate dehydrogenase, no enzyme of the tricarboxylic (TCA) cycle has been conclusively demonstrated in the mollicutes (Lanham et al., 1980; Manolukas et al., 1988; O'Brien et al., 1981). In the present study, however, isocitrate dehydrogenase (NADPH-dependent) (ICDH) was detected in extracts of *An. intermedium*, distinguishing it from all other mollicutes. The biological significance of this is uncertain because citrate synthase was not detected in *An. intermedium;* this enzyme normally precedes ICDH in the TCA cycle. Citrate synthase generates isocitrate, the substrate for ICDH; ICDH then converts isocitrate to

α-ketoglutarate. α-Ketoglutarate is a reactant in the aspartate aminotransferase reaction, an enzyme that *An. intermedium* possesses. On the other hand, if *An. intermedium* was in an environment where isocitrate was available, the organism could oxidize this substrate, concomitantly generating NADPH. Because *An. intermedium* lacks the oxidative shunt portion of the pentose phosphate pathway, it cannot generate biosynthetically important NADPH in this manner. Thus, the potential generation of NADPH by ICDH takes on added importance.

The ostensive occurrence of ICDH but not citrate synthase in *An. intermedium* implies that this organism may have once had the genetic information for the production of citrate synthase, but that this genetic information may have been lost during the genome reductions that are proposed to have occurred during the degenerative evolution of wall-less prokaryotes (Rogers et al., 1985; Sladek, 1986). The paucity of metabolic capabilities of mollicutes has been explained in part by the proposed genome reductions in these organisms and their resulting limited genomic content (Berry et al., 1987; Davis et al., 1988; Williams and Pollack, 1988). DeSantis et al. (1989) have suggested that the presence of Glc-6-*P* isomerase and the absence of PFK and aldolase in nonfermentative mycoplasmas may be the result of the retention of the genetic information for Glc-6-*P* isomerase from a phylogenetic progenitor with a more complete glycolytic capability. An analogous situation may exist with ICDH in *An. intermedium*.

Another distinction of the anaerobic mollicutes was the presence of PEP carboxykinase (EC 4.1.1.31/49) in *An. intermedium;* the activity was dependent on the presence of ADP (or GDP) and fructose-1,6-*P*$_2$ (Fru-1,6-*P*$_2$). Furthermore, the enzyme was active at pH 6.6 (100m*M* imidazole) but not at 7.8 (50m*M* Tris). Manolukas et al. (1988) did not detect PEP carboxykinase in ten mycoplasmas and acholeplasmas; these investigators performed the assays at pH 7.4 (100m*M* HEPES) in the absence of Fru-1,6-*P*$_2$. However, Manolukas et al. (1988, 1989) detected PEP carboxylase (EC 4.1.1.31) in *Acholeplasma* spp. but not in *Mycoplasma* spp. The PEP carboxylase activity was stimulated by Fru-1,6-*P*$_2$, but it was active in the absence of ribonucleotides. Thus, acholeplasmas and *An. intermedium,* unlike other mollicutes, are able to carboxylate PEP to oxaloacetate, although apparently by different mechanisms.

The purine metabolism of *As. anaerobium,* examined in the present study, and *An. intermedium,* examined by McElwain et al. (1988), was similar to that of the other mollicutes (McElwain et al., 1988). The strictly anaerobic mollicutes are able to interconvert purine bases, (deoxy)ribonucleosides, and (deoxy)ribomononucleotides. The nucleoside kinase activity of *As. anaerobium* was active with PP$_i$ but not with ATP; acholeplasmas, spiroplasmas, *An. intermedium,* and some strains of *M. hominis* have PP$_i$-nucleoside kinase activity (McElwain et al., 1988; Pollack et al., 1989). PP$_i$-nucleoside kinase is unique to the *Mollicutes.*

The pyrimidine metabolism of *As. anaerobium* was examined in the present study and compared with the enzymes of pyrimidine metabolism detected by McElwain et al. (1988) in *An. intermedium*. Whereas *An. intermedium* possessed both dCyd deaminase (Williams, Petzel, and Pollack, unpublished result) and dCMP deaminase (McElwain et al., 1988), neither enzyme was detected in *As. anaerobium* (this study). However, the two strictly anaerobic mollicutes were similar to one another in that they lacked dCyd kinase (McElwain et al. 1988; this study). The inability to form dCMP from dCyd distinguishes *An. intermedium* and *As. anaerobium* from the rest of the mollicutes (McElwain et al., 1988). Both organisms, however, have dCMP kinase activity (McElwain et al., 1988; Williams, Petzel, and Pollack, unpublished result). Thus, if the strictly anaerobic mollicutes are able to incorporate exogenous deoxyribomononucleotides [as *M. mycoides* subsp. *mycoides* can (Neale et al., 1984)], then they would be capable of dCyd nucleotide synthesis by the salvage pathway.

Enzymological Evidence Supportive of the Phylogenetic Relationships among *Mollicutes* and their Walled Relatives

Subgroups among the walled relatives

Six walled bacteria (*Clostridium innocuum, C. ramosum, Erysipelothrix rhusiopathiae, Lactobacillus catenaformis, L. vitulinus,* and *Streptococcus pleomorphus*) that are proposed to be phylogenetically related to the mollicutes based on rRNA-sequence analyses (Ludwig et al.,

1988; Weisburg et al., 1989; Woese, 1985; Woese et al., 1980, 1985) were examined in the present study for PP_i-dependent enzymes. PP_i-dependent enzymes are uncommon, and in most bacteria, ATP serves as the phosphoryl donor in analogous reactions instead of PP_i (Reeves, 1987; Wood, 1985). PP_i-dependent enzymes occur more frequently among the *Mollicutes*, however.

The first report of a PP_i-dependent enzyme in the *Mollicutes* was by Beaman and Pollack (1984), who detected PEP carboxytransphosphorylase (PEP-CTP; EC 4.1.1.38) in *A. laidlawii*. However, workers from the same laboratory could not confirm this finding (Manolukas et al., 1988). Tryon and Pollack (1984) reported the occurrence of another PP_i-dependent enzyme in the *Mollicutes*. PP_i-dependent nucleoside kinase activity was detected in *A. laidlawii;* this was the first report of a PP_i-dependent nucleoside kinase in any organism. PP_i-nucleoside kinase has subsequently been detected in spiroplasmas, the two strictly anaerobic mollicutes, and some strains of *M. hominis* (McElwain et al., 1988; Pollack et al., 1989; this study). Pollack and Williams (1986) demonstrated that a third PP_i-dependent enzyme, PP_i-PFK, was present in *Acholeplasma* spp. This enzyme has not been detected in mycoplasmas or spiroplasmas (DeSantis et al., 1989; Lake-Bullock et al., 1989; Pollack et al., 1989), but it was observed in *An. intermedium* and *An. bactoclasticum* (this study). Thus, two and possibly three PP_i-dependent enzymes have been detected among the mollicutes.

Because of this prominent occurrence of PP_i-enzymes in the *Mollicutes*, I decided to examine their walled relatives for four PP_i-dependent enzymes: PP_i-PFK, PPDK, PEP-CTP, and PP_i-acetate kinase. Differences in enzymic cofactor specificity (in the present study, PP_i *versus* ATP) is one criterion used by Jensen (1985) to examine the evolution of bacteria and their metabolic pathways. A search for these uncommon PP_i-dependent enzymes in a group of phylogenetically related bacteria might be fruitful as a phenotypic indicator of their relatedness. Furthermore, if the occurrence of one or more of these PP_i-dependent enzymes among members of this phylogenetic unit was not uniform, the distribution of the enzyme(s) could be compared with the subdivisions of the larger phylogenetic unit to determine if there was a correlation.

Weisburg et al. (1989) used 16S-rRNA analysis to subdivide the six walled relatives of the mollicutes into smaller clusters. *C. innocuum* and *S. pleomorphus* formed one cluster. *C. ramosum* clustered with *L. catenaformis* and *L. vitulinus*, with the lactobacilli being more closely related to each other than to the clostridium. *E. rhusiopathiae* did not cluster with any of the other walled relatives.

PP_i-PFK was detected in the cluster formed by *C. innocuum* and *S. pleomorphus*, but not in the cluster of *C. ramosum* and the two lactobacilli (this latter group had only ATP-PFK); *E. rhusiopathiae* had PP_i-PFK activity. All of the walled relatives except *E. rhusiopathiae* had PPDK activity. Thus, PPDK is proposed to be a phenotypic indicator

of the general phylogenetic relatedness of this group, but the enzyme cannot be used to substantiate the subdivisions of this group. PEP-CTP was detected by two methods in *C. innocuum* and *S. pleomorphus*, which form one cluster, and in *C. ramosum*. The two lactobacilli, which are a subcluster within the *C. ramosum* cluster, had low PEP CTP activity in the assay for the appearance of PP_i; no activity was detected with the assay for oxaloacetate. The enzyme was not detected in *E. rhusiopathiae*. None of the bacteria examined in the present study possessed PP_i-acetate kinase activity. This enzyme has only been reported in the protozoan *Entamoeba histolytica* (Wood, 1985) and in Gram-negative, sulfate-reducing anaerobes (Liu and Peck, 1981). Since their initial report, however, Liu and Peck (cited by Wood, 1985) have encountered difficulties in confirming the presence of PP_i-acetate kinase in the sulfate-reducing anaerobes. Finally, all the walled phylogenetic relatives of the mollicutes, except *E. rhusiopathiae*, possessed pyruvate kinase. In four of these species, Glc-6-*P* was a better activator of the enzyme than Fru-1,6-P_2. This pattern of allosteric activation of pyruvate kinase has been reported in only a few bacteria (Smart and Pritchard, 1979; Yamada and Carlsson, 1975); in most other bacteria, Fru-1,6-P_2 is a primary activator (Sanwal, 1970; Smart and Pritchard, 1979). Thus, the occurrence of this uncommon allosteric pattern in these bacteria is an indication of their relatedness.

In summary, *C. innocuum* and *S. pleomorphus*, which formed one cluster, were similar in regards to all the PP_i-dependent enzymes; both

had PP_i-PFK, PPDK, and PEP-CTP. Each of the members of the second cluster (*C. ramosum*, *L. catenaformis*, and *L. vitulinus*) had PPDK and ATP-PFK but not PP_i-PFK. The two lactobacilli, which are more closely related to one another than to *C. ramosum*, could be distinguished from this clostridium in regards to PEP-CTP and pyruvate kinase. PEP-CTP was detected by two methods in *C. ramosum*, but it was detected, at low levels, only by the PP_i assay in the two lactobacilli. The pyruvate kinase activity of the lactobacilli were activated to a greater degree by AMP than by Glc-6-*P;* the opposite was true with *C. ramosum*. *E. rhusiopathiae* was distinct from the other five walled relatives of the mollicutes in three properties: (i) neither PPDK nor pyruvate kinase was detected in this organism; (ii) PP_i-PFK but not ATP-PFK was detected in this organism; the other species determined to have PP_i-PFK activity in the present study also possessed putative ATP-PFK (see Results and Discussion in Section II); and (iii) PEP-CTP was not detected in *E. rhusiopathiae*.

PP_i-dependent enzymes are not the only enzymes that are potentially useful in substantiating the phylogenetic relatedness of the mollicutes and certain walled bacteria. Berry et al. (1987) characterized the pathway of aromatic amino acid biosynthesis in *A. laidlawii*. The pattern of allosteric regulation of prephenate dehydratase was supportive of the proposed phylogenetic relationship between mollicutes and the low-G + C, Gram-positive bacteria. Another enzyme of potential interest was described by Akao et al. (1988) in *C. innocuum*. This clostridium

possessed a novel enzyme, 3α-hydroxyglycyrrhetinate dehydrogenase, that converts 3-ketoglycyrrhetinate to 3α-hydroxyglycyrrhetinate. These compounds are metabolites of glycyrrhizin, the active component of liquorice, an oriental medicine. If this enzyme were found in mollicutes, it could provide evidence of their phylogenetic relatedness to *C. innocuum*. Unfortunately, the substrate is not commercially available.

Subgroups among the mollicutes

When compared with the enzymic characteristics reported in the literature for the other mollicutes, some of the enzymic characteristics of the strictly anaerobic mollicutes determined in the present study correlated with the phylogenetic subdivisions of the mollicutes proposed by Woese and colleagues using rRNA analyses (Rogers et al., 1985; Weisburg et al., 1989).

Rogers et al. (1985) proposed that the acholeplasmas represent the first branch of wall-less organisms to diverge from the walled progenitors. Anaeroplasmas split from the acholeplasmas somewhere close to the original divergence, although the exact placement of the anaeroplasma branch is uncertain (Rogers et al., 1985). This close association of the acholeplasmas and anaeroplasmas was confirmed by Weisburg et al. (1989), who reported that acholeplasmas and anaeroplasmas form a monophyletic subgroup among the mollicutes and their walled relatives. In regard to enzymic characteristics, *A. laidlawii* and

An. intermedium differed with respect to the enzymes of the oxidative shunt portion of the pentose phosphate pathway and the presence of ICDH. However, they were similar to one another (and distinct from the *Mycoplasmatales*) in that they possessed hexokinase, PP_i-PFK, and they were able to carboxylate PEP to oxaloacetate (DeSantis et al., 1989; Manolukas et al., 1988, 1989; this study). Furthermore, McElwain et al. (1988) reported that *A. laidlawii* and *An. intermedium* could not be distinguished by their purine and pyrimidine metabolism. Examination of the biosynthetic pathway for aromatic amino acid biosynthesis in the anaeroplasmas could potentially provide further evidence of the relatedness of acholeplasmas and anaeroplasmas.

As. anaerobium was determined in the present study to posssess PPDK; this PP_i-dependent enzyme has not been detected in any other mollicute (Manolukas et al., 1988). This characteristic of *As. anaerobium* may be a reflection of its unique phylogenetic status among the mollicutes (Weisburg et al., 1989).

In concluding this discussion, I would like to address a taxonomic question that I believe will present difficulties in the future. Binder and Kirchhoff (1988) recently described the isolation of sterol-nonrequiring, "obligately anaerobic" mollicutes from the intestines of swine. These isolates were oxyduric; they survived several hours in the air, but they required anaerobic conditions for growth. Thus, these organisms would be classified as obligate anaerobes by the definition of Hungate (1985) because they require anaerobic conditions for growth,

although they survive exposure to oxygen. Binder and Kirchhoff (1988) proposed that the anaerobic nature and sterol independence of their isolates warrented the creation of a new species in the genus *Asteroleplasma*. However, *As. anaerobium* and *Anaeroplasma* spp. are not obligate anaerobes (*sensu* Hungate); they are killed by even brief exposure to O_2 (Robinson, 1979; Robinson and Freundt, 1987). Thus, they would be classified as oxylabile anaerobes by Hungate (1985) or strict anaerobes by Gottschalk (1986). This terminology is crucial, because Robinson and Freundt (1987) used the term "obligately anaerobic" in the descriptions of the order *Anaeroplasmatales* and family *Anaeroplasmataceae*. Thus, both Robinson and Freundt (1987) and Binder and Kirchhoff (1988) utilized the term "obligately anaerobic" in the descriptions of the organisms involved, but the term was used in a different sense in the two reports.

Because of this disparity between the natures of anaerobiosis as described by Robinson and Freundt (1987) and Binder and Kirchhoff (1988), the isolates of Binder and Kirchhoff cannot be classified in the genus *Asteroleplasma* or even in the order *Anaeroplasmatales* as they are currently described. Therefore, the following recommendations are made. First, standard definitions of terms for different degrees of anaerobiosis should be adopted and utilized in the descriptions of *Mollicutes*.

Second, DNA-DNA hybridization studies with *As. anaerobium* and the swine isolates should be conducted to determine the degree of

relatedness. In addition, the G + C content of the DNA of the swine isolates should be determined. *As. anaerobium* has a G + C content of 40.2 mol%; except for *M. pneumoniae*, all other mollicutes have a G + C content of less than 36 mol% (Razin and Freundt, 1984). Because *As. anaerobium* is phylogenetically distinct among mollicutes (Weisburg et al., 1989), a phylogenetic comparison of the 16S-rRNA sequences of *As. anaerobium* and the swine isolates would be informative.

Third, and finally, if Binder and Kirchhoff wish to classify their isolates in the order *Anaeroplasmatales*, the description of this order must be changed such that inclusion of an obligately anaerobic mollicute (*sensu* Hungate) would be appropriate. A distinction between obligately anaerobic organisms (*sensu* Hungate) and strictly anaerobic organisms (*sensu* Gottschalk) should be maintained at the genus or family level.

If such a radical revision of the order *Anaeroplasmatales* is proposed, the inclusion of other mollicutes would be warrented. For example, *Mycoplasma muris* requires anaerobic conditions for growth (McGarrity et al., 1983); it is apparently an obligate anaerobe (*sensu* Hungate). The preferred, initial step before making radical taxonomic changes of this sort would be a genetic comparison of Binder and Kirchhoff's (1988) isolates with members of the *Anaeroplasmatales* and *Acholeplasmatales*. The examination of the swine isolates for PP_i-PFK and PPDK would also be informative.

REFERENCES CITED

Akao, T., T. Akao, M. Hattori, T. Namba, and K. Kobashi. 1988. Purification and properties of 3α-hydroxyglycyrrhetinate dehydrogenase of *Clostidium innocuum* from human intestine. J. Biochem. 103:504-507.

Atobe, H., J. Watabe, and M. Ogata. 1983. *Acholeplasma parvum,* a new species from horses. Int. J. Syst. Bacteriol. 33:344-349.

Ball, H. J., S. D. Neill, and L. R. Reid. 1982. Use of arginine aminopeptidase activity in characterization of arginine-utilizing mycoplasmas. J. Clin. Microbiol. 15:28-34.

Barile, M. F., J. M. Bové, J. M. Bradbury, G. H. Cassell, W. A. Clyde, Jr., G. S. Cottew, and P. Whittlestone. 1985. Current status on control of mycoplasmal diseases of man, animals, plants and insects. Bull. 'Inst. Pasteur 83:339-373.

Barile, M. F., and S. Razin (eds.). 1979. The mycoplasmas. Volume I. Cell biology. Academic Press, Inc., New York.

Bastian, F. O., R. A. Jennings, and W. A. Gardner. 1987. Antiserum to scrapie-associated fibril protein cross-reacts with *Spiroplasma mirum* fibril proteins. J. Clin. Microbiol. 25:2430-2431.

Beaman, K. D., and J. D. Pollack. 1981. Adenylate energy charge in *Acholeplasma laidlawii.* J. Bacteriol. 146:1055-1058.

Beaman, K. D., and J. D. Pollack. 1983. Synthesis of adenylate nucleotides by mollicutes (mycoplasmas). J. Gen. Microbiol. 129:3103-3110.

Beaman, K. D., and J. D. Pollack. 1984. Enzymatic assimilation of ^{14}C from $NaH^{14}CO_3$ by extracts of *Acholeplasma laidlawii* B-PG9. Yale J. Biol. Med. 57:897 (Abstr.).

Berry, A., S. Ahmad, A. Liss, and R. A. Jensen. 1987. Enzymological features of aromatic amino acid biosynthesis reflect the phylogeny of mycoplasmas. J. Gen. Microbiol. 133:2147-2154.

Binder, A., and H. Kirchhoff. 1988. Isolation of anaerobic mollicutes from the intestine of swine. Vet. Microbiol. 17:151-158.

Board of directors. 1983. Caprine and ovine mycoplasmas and anaeroplasmas. Pages 14-16 *in* International research programme on comparative mycoplasmology, 1980-1982. Report of consultations, Tokyo, Japan, 8th-9th September 1982. International Organization for Mycoplasmology, Jerusalem.

Bové, J. M., and J. G. Tully (eds.). 1984. Pathogenicity of mycoplasmas. Issue number 1 of Ann. 'Inst. Pasteur (Paris) 135A:7-179.

Castrejon-Diez, J., T. N. Fisher, and E. Fisher, Jr. 1962. Acetokinase reaction in several pleuropneumoniae-like organisms. Biochem. Biophys. Res. Commun. 9:416-420.

Castrejon-Diez, J., T. N. Fisher, E. Fisher, Jr. 1963. Glucose metabolism of two strains of *Mycoplasma laidlawii*. J. Bacteriol. 86:627-636.

Chanock, R. M., L. Hayflick, and M. F. Barile. 1962. Growth on artificial medium of an agent associated with atypical pneumonia and its identification as a PPLO. Proc. Natl. Acad. Sci. USA 48:41-49.

Choules, G. L., and W. R. Gray. 1971. Peptidase activity in the membranes of *Mycoplasma laidlawii*. Biochem. Biophys. Res. Commun. 45:849-855.

Christiansen, C., E. A. Freundt, and I. M. Robinson. 1986. Genome size and deoxyribonucleic acid base composition of *Anaeroplasma abactoclasticum*, *Anaeroplasma bactoclasticum*, and a sterol-nonrequiring anaerobic mollicute. Int. J. Syst. Bacteriol. 36:483-485.

Clementz, T., A. Christiansson, and A. Wieslander. 1987. Membrane potential, lipid regulation and adenylate energy charge in acyl chain modified *Acholeplasma laidlawii*. Biochim. Biophys. Acta 898:299-307.

Cocks, B. G., F. A. Brakes, A. Mitchell, and L. R. Finch. 1985. Enzymes of intermediary carbohydrate metabolism in *Ureaplasma urealyticum* and *Mycoplasma mycoides* subsp. *mycoides*. J. Gen. Microbiol. 131:2129-2135.

Cocks, B. G, R. Youil, and L. R. Finch. 1988. Comparison of enzymes of nucleotide metabolism in two members of the *Mycoplasmataceae* family. Int. J. Syst. Bacteriol. 38:273-278.

Constantopoulos, G., and G. J. McGarrity. 1987. Activities of oxidative enzymes in mycoplasmas. J. Bacteriol. 169:2012-2016.

Cruden, D. L., W. E. Durbin, and A. J. Markovetz. 1983. Utilization of PP_i as an energy source by a *Clostridium* sp. Appl. Environ. Microbiol. 46:1403-1408.

Davis, J. R., Jr., A. Dokun, J. Liburd, and I. Villanueva. 1989. Phosphoenolpyruvate, pyruvate, malate and oxaloacetate metabolism; and the absence of a tricarboxylic acid cycle in *Ureaplasma diversum* and ovine ureaplasmas. Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, G-38, p. 154.

Davis, J. W., Jr., J. Manolukas, B. Capo, J. Espinoza, and J. D. Pollack. 1988. Pyruvate metabolism and the absence of a TCA cycle in *Ureaplasma urealyticum*. Abstr. Seventh Congr. Int. Organ. Mycoplasmol. 1988, P-42.

Delisle, G. J. 1977. Multiple forms of urease in cytoplasmic fractions of *Ureaplasma urealyticum*. J. Bacteriol. 130:1390-1392.

DeSantis, D., V. V. Tryon, and J. D. Pollack. 1989. Metabolism of mollicutes: The Embden-Meyerhof-Parnas pathway and the hexose monophosphate shunt. J. Gen. Microbiol. 135:683-691.

Dienes, L. 1938. Further observations on the L organism of Klieneberger. Proc. Soc. Exp. Biol. Med. 39:365-367.

Dienes, L., and G. Edsall. 1937. Observations on the L-organism of Klieneberger. Proc. Soc. Exp. Biol. Med. 36:740-744.

Dienes, L., and H. J. Weinberger. 1951. The L forms of bacteria. Bacteriol. Rev. 15:245-288.

Doolittle, W. F. 1978. Genes in pieces: Were they ever together? Nature 272:581-582.

Edward, D. G. ff. 1950. An investigation of pleuropneumonia-like organisms isolated from the bovine genital tract. J. Gen. Microbiol. 4:4-15.

Edward, D. G. ff., and E. A. Freundt. 1970. Amended nomenclature for strains related to *Mycoplasma laidlawii*. J. Gen. Microbiol. 62:1-2.

Egan, W., M. Barile, and S. Rottem. 1986. ^{31}P-NMR studies of *Mycoplasma gallisepticum* cells using continuous perfusion technique. FEBS Lett. 204:373-376.

Elton, R. A. 1973. The relationship of DNA base composition and individual protein composition in micro-organisms. J. Mol. Evol. 2:263-276.

Fenske, J. D., and G. E. Kenny. 1976. Role of arginine deiminase in growth of *Mycoplasma hominis*. J. Bacteriol. 126:501-510.

Freundt, E. A., and S. Razin. 1984. Genus I. Mycoplasma. Pages 742-770 *in* N. R. Krieg and J. G. Holt (eds.), Bergey's manual of systematic bacteriology. Volume 1. Williams & Wilkins, Baltimore.

Freundt, E. A., R. F. Whitcomb, M. F. Barile, S. Razin, and J. G. Tully. 1984. Proposal for elevation of the family *Acholeplasmataceae* to ordinal rank: *Acholeplasmatales*. Int. J. Syst. Bacteriol. 34:346-349.

Gabridge, M. G., and Y. Dee Barden Stahl. 1978. Role of adenine in the pathogenesis of *Mycoplasma pneumoniae* infections of tracheal epithelium. Med. Microbiol. Immunol. 165:43-55.

Gaisser, H.-D., J. de Vries, H. van der Goot, and H. Timmerman. 1987. Inhibition of NADH oxidase and lactate dehydrogenase of *Mycoplasma gallisepticum* by copper complexes of 2,2'-bipyridyl analogues. Biochem. Pharmacol. 36:3237-3241.

Gottschalk, G. 1986. Bacterial metabolism. Second edition. Springer-Verlag, New York.

Hackett, K. J., D. E. Lynn, D. L. Williamson, A. S. Ginsberg, and R. F. Whitcomb. 1986. Cultivation of the *Drosophila* sex-ratio spiroplasma. Science 232:1253-1255.

Hahn, R. G., and G. E. Kenny. 1974. Differences in arginine requirement for growth among arginine-utilizing *Mycoplasma* spp. J. Bacteriol. 117:611-618.

Hamet, M., C. Bonissol, and P. Cartier. 1980. Activities of enzymes of purine and pyrimidine metabolism in nine mycoplasma species. Pages 231-235 *in* A. Rapado, R. W. E. Watts, and C. H. M. M. De Bruyn (eds.), Purine metabolism in man - III: Biochemical, immunological, and cancer research, (Adv. Exp. Med. Biol. 122B:231-235). Plenum Press, New York.

Hayflick, L. 1969. Fundamental biology of the class Mollicutes, order Mycoplasmatales. Pages 15-47 *in* L. Hayflick (ed.), The mycoplasmatales and the L-phase of bacteria. Appleton-Century-Crofts, New York.

Herring, P. K., and J. D. Pollack. 1974. Utilization of [1-^{14}C]acetate in the synthesis of lipids by acholeplasmas. Int. J. Syst. Bacteriol. 24:73-78.

Holländer, R., G. Wolf, and W. Mannheim. 1977. Lipoquinones of some bacteria and mycoplasmas, with considerations on their functional significance. Antonie Leeuwenhoek J. Microbiol. Serol. 43:177-185.

Howard, C. J., and R. N. Gourlay. 1982. Proposal for a second species within the genus *Ureaplasma*, *Ureaplasma diversum* sp. nov. Int. J. Syst. Bacteriol. 32:446-452.

Hungate, R. E. 1966. The rumen and its microbes. Academic Press, Inc., New York.

Hungate, R. E. 1970. Interrelationships in the rumen microbiota. Pages 292-305 *in* A. T. Phillipson (ed.), Physiology of digestion and metabolism in the ruminant (Proceedings of the Third International Symposium). Oriel Press, Ltd., Newcastle upon Tyne.

Hungate, R. E. 1985. Anaerobic biotransformations of organic matter. Pages 39-95 *in* E. R. Leadbetter and J. S. Poindexter (eds.), Bacteria in nature. Volume 1. Bacterial activities in perspective. Plenum Press, New York.

Igwegbe, E. C. K., and C. Thomas. 1978. Occurrence of enzymes of arginine dihydrolase pathway in *Spiroplasma citri*. J. Gen. Appl. Microbiol. 24:261-269.

Jensen, R. A. 1985. Biochemical pathways in prokaryotes can be traced backward through evolutionary time. Mol. Biol. Evol. 2:92-108.

Jordan, F. T. W. 1979. Avian mycoplasmas. Pages 1-48 *in* J. G. Tully and R. F. Whitcomb (eds.), The mycoplasmas. Volume II. Human and animal mycoplasmas. Academic Press, Inc., New York.

Jost, C. 1979. *Anaeroplasma bactoclasticum* extracellular amylase. Ph.D. dissertation, Iowa State University Library, Ames. (Diss. Abstr. Int. B Sci. Eng. 40:2056-B, 1979).

Kahane, I., S. Razin, and A. Muhlrad. 1978. Possible role of acetate kinase in ATP generation in *Mycoplasma hominis* and *Acholeplasma laidlawii*. FEMS Microbiol. Lett. 3:143-145.

Kirchhoff, H., R. Rosengarten, W. Lotz, M. Fischer, and D. Lopatta. 1984. Flask-shaped mycoplasmas: Properties and pathogenicity for man and animals. Isr. J. Med. Sci. 20:848-853.

Klieneberger, E. 1935. The natural occurrence of pleuropneumonia-like organisms in apparent symbiosis with *Streptobacillus moniliformis* and other bacteria. J. Pathol. Bacteriol. 40:93-105.

Kotani, H., D. Phillips, and G. J. McGarrity. 1986. Malignant transformation of NIH-3T3 and CV-1 cells by a helical mycoplasma, *Spiroplasma mirum*, strain SMCA. In Vitro (Rockville) 22:756-762.

Laidlaw, P. P., F.R.S., and W. J. Elford. 1936. A new group of filterable organisms. Proc. R. Soc. Lond. B Biol. Sci. 120:292-303.

Lake-Bullock, M. H. V., W. T. Blevins, S. K. McGlynn, and T. L. Rhoads. 1989. Pathways of glucose catabolism in species of *Spiroplasma*. Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, G-39, p. 155.

Langworthy, T. A., W. R. Mayberry, P. F. Smith, and I. M. Robinson. 1975. Plasmalogen composition of *Anaeroplasma*. J. Bacteriol. 122:785-787.

Lanham, S. M., R. M. Lemcke, C. M. Scott, and J. M. Grendon. 1980. Isozymes in two species of *Acholeplasma*. J. Gen. Microbiol. 117:19-31.

Lecce, J. G., and H. E. Morton. 1954. Metabolic studies on three strains of pleuropneumonia-like organisms isolated from man. J. Bacteriol. 67:62-68.

Lemcke, R. M. 1979. Equine mycoplasmas. Pages 177-189 *in* J. G. Tully and R. F. Whitcomb (eds.), The mycoplasmas. Volume II. Human and animal mycoplasmas. Academic Press, Inc., New York.

Liu, C.-L., and H. D. Peck, Jr. 1981. Comparative bioenergetics of sulfate reduction in *Desulfovibrio* and *Desulfotomaculum* spp. J. Bacteriol. 145:966-973.

Ludwig, W., M. Weizenegger, R. Kilpper-Bälz, and K. H. Schleifer. 1988. Phylogenetic relationships of anaerobic streptococci. Int. J. Syst. Bacteriol. 38:15-18.

Macy, J. M., L. G. Ljungdahl, and G. Gottschalk. 1978. Pathway of succinate and propionate formation in *Bacteroides fragilis*. J. Bacteriol. 134:84-91.

Mandelbaum-Shavit, F., and I. Kahane. 1988. Activity of dihydrofolate reductase in the mollicutes *Acholeplasma laidlawii* and *Mycoplasma gallisepticum* and their suseptibility to antifolates. Curr. Microbiol. 17:351-354.

Maniloff, J. 1989. Anomalous values of *Mycoplasma* genomes sizes (sic) determined by pulse-field gel electrophoresis. Nucleic Acids Res. 17:1268.

Manolukas, J. T., M. F. Barile, D. K. F. Chandler, and J. D. Pollack. 1988. Presence of anaplerotic reactions and transamination, and the absence of the tricarboxylic acid cycle in mollicutes. J. Gen. Microbiol. 134:791-800.

Manolukas, J. T., M. V. Williams, and J. D. Pollack. 1989. The anaplerotic phosphoenolpyruvate carboxylase of the tricarboxylic acid deficient *Acholeplasma laidlawii* B-PG9. J. Gen. Microbiol. 135:251-256.

Masover, G. K., S. Razin, and L. Hayflick. 1977. Localization of enzymes in *Ureaplasma urealyticum* (T-strain mycoplasma). J. Bacteriol. 130:297-302.

McCoy, R. E. 1984. Mycoplasma-like organisms of plants and invertebrates. Pages 792-793 *in* N. R. Krieg and J. G. Holt (eds.), Bergey's manual of systematic bacteriology. Volume 1. Williams & Wilkins, Baltimore.

McElhaney, R. N. 1984. The structure and function of the *Acholeplasma* plasma membrane. Biochim. Biophys. Acta 779:1-42.

McElhaney, R. N. 1986. Modifications of membrane lipid structure and their influence on cell growth, passive permeability, and enzymatic and transport activities in *Acholeplasma laidlawii* B. Biochem. Cell Biol. 64:58-65.

McElwain, M. C., and J. D. Pollack. 1987. Synthesis of deoxyribomononucleotides in mollicutes: Dependence on deoxyribose-1-phosphate and PP_i. J. Bacteriol. 169:3647-3653.

McElwain, M. C., D. K. F. Chandler, M. F. Barile, T. F. Young, V. V. Tryon, J. W. Davis, Jr., J. P. Petzel, C.-J. Chang, M. V. Williams, and J. D. Pollack. 1988. Purine and pyrimidine metabolism in *Mollicutes* species. Int. J. Syst. Bacteriol. 38:417-423.

McGarrity, G. J., D. L. Rose, V. Kwiatkowski, A. S. Dion, D. M. Phillips, and J. G. Tully. 1983. *Mycoplasma muris*, a new species from laboratory mice. Int. J. Syst. Bacteriol. 33:350-355.

McGee, Z. A., M Rogul, and R. G. Witter. 1967. Molecular genetic studies of relationships among mycoplasma, L-forms and bacteria. Ann. N. Y. Acad. Sci. 143:21-30.

Mitchell, A., and L. R. Finch. 1977. Pathway of nucleotide biosynthesis in *Mycoplasma mycoides* subsp. *mycoides*. J. Bacteriol. 130:1047-1054.

Morowitz, H. J. 1984. The completeness of molecular biology. Isr. J. Med. Sci. 20:750-753.

Morowitz, H. J., and T. M. Terry. 1969. Characterization of the plasma membrane of *Mycoplasma laidlawii*. V. Effects of selective removal of protein and lipid. Biochim. Biophys. Acta 183:276-294.

Muhlrad, A., I. Peleg, J. A. Robertson, I. M. Robinson, and I. Kahane. 1981. Acetate kinase activity in mycoplasmas. J. Bacteriol. 147:271-273.

Neale, G. A. M., A. Mitchell, and L. R. Finch. 1983. Enzymes of pyrimidine deoxyribonucleotide metabolism in *Mycoplasma mycoides* subsp. *mycoides*. J. Bacteriol. 156:1001-1005.

Neale, G. A. M., A. Mitchell, and L. R. Finch. 1984. Uptake and utilization of deoxynucleotide 5'-monophosphates by *Mycoplasma mycoides* subsp. *mycoides*. J. Bacteriol. 158:943-947.

Neimark, H. 1979. Phylogenetic relationships between mycoplasmas and other prokaryotes. Pages 43-61 *in* M. F. Barile and S. Razin (eds.), The mycoplasmas. Volume I. Cell biology. Academic Press, Inc., New York.

Neimark, H., and J. London. 1982. Origins of the mycoplasmas: Sterol-nonrequiring mycoplasmas evolved from streptococci. J. Bacteriol. 150:1259-1265.

Neimark, H., and M. C. Tung. 1973. Properties of a fructose-1,6-diphosphate-activated lactate dehydrogenase from *Acholeplasma laidlawii* type A. J. Bacteriol. 114:1025-1033.

Neimark, H. C., and M. J. Pickett. 1960. Products of glucose metabolism by pleuropneumonialike (sic) organisms. Ann. N.Y. Acad. Sci. 79:531-537.

Nur, I., G. Glaser, and S. Razin. 1986. Free and integrated plasmid DNA in spiroplasmas. Curr. Microbiol. 14:169-176.

O'Brien, S. J., J. M. Simonson, M. W. Grabowski, and M. F. Barile. 1981. Analysis of multiple isozyme expression among twenty-two species of *Mycoplasma* and *Acholeplasma*. J. Bacteriol. 146:222-232.

O'Brien, W. E., S. Bowien, and H. G. Wood. 1975. Isolation and characterization of a pyrophosphate-dependent phosphofructokinase from *Propionibacterium shermanii*. J. Biol. Chem. 250:8690-8695.

Patterson, A., C. Stevens, R. M. Cody, and R. T. Gudauskas. 1985. Differential amino acid utilization by spiroplasmas and the effect on growth kinetics. J. Gen. Appl. Microbiol. 31:499-505.

Petes, T. D., and C. W. Hill. 1988. Recombination between repeated genes in microorganisms. Annu. Rev. Genet. 22:147-168.

Pierce, C. H. 1942. *Streptobacillus moniliformis*, its associated L_1 form, and other pleuropneumonia-like organisms. J. Bacteriol. 43:780 (Abstr.).

Pollack, J. D. 1975. Localization of reduced nicotinamide adenine dinucleotide oxidase activity in *Acholeplasma* and *Mycoplasma* species. Int. J. Syst. Bacteriol. 25:108-113.

Pollack, J. D. 1978. Differentiation of *Mycoplasma* and *Acholeplasma*. Int. J. Syst. Bacteriol. 28:425-426.

Pollack, J. D. 1979. Respiratory pathways and energy-yielding mechanisms. Pages 187-211 *in* M. F. Barile and S. Razin (eds.), The mycoplasmas. Volume I. Cell biology. Academic Press, Inc., New York.

Pollack, J. D. 1986. Metabolic distinctiveness of ureaplasmas. Pediatr. Infect. Dis. J. 5:S305-S307.

Pollack, J. D., and P. J. Hoffmann. 1982. Properties of the nucleases of mollicutes. J. Bacteriol. 152:538-541.

Pollack, J. D., and M. V. Williams. 1986. PP_i-dependent phosphofructotransferase (phosphofructokinase) activity in the mollicutes (mycoplasma) *Acholeplasma laidlawii*. J. Bacteriol. 165:53-60.

Pollack, J. D., A. J. Merola, M. Platz, and R. L. Booth, Jr. 1981. Respiration-associated components of *Mollicutes*. J. Bacteriol. 146:907-913.

Pollack, J. D., V. V. Tryon, and K. D. Beaman. 1983. The metabolic pathways of *Acholeplasma* and *Mycoplasma:* An overview. Yale J. Biol. Med. 56:709-716.

Pollack, J. D., K. D. Beaman, and J. A. Robertson. 1984*a*. Synthesis of lipids from acetate is not characteristic of *Acholeplasma* or *Ureaplasma* species. Int. J. Syst. Bacteriol. 34:124-126.

Pollack, J. D., K. D. Beaman, and V. V. Tryon. 1984*b*. New emerging patterns of NADH oxidase localization and lipid synthesis in mollicutes. Yale J. Biol. Med. 57:891 (Abstr.).

Pollack, J. D., M. C. McElwain, D. DeSantis, J. Manolukas, J. G. Tully, C.-J. Chang, R. A. Whitcomb, K. J. Hackett, and M. V. Williams. 1989. The metabolism of spiroplasmas. Int. J. Syst. Bacteriol., submitted for publication. (See also Abstr. Seventh Congress Int. Organ. Mycoplasmol. 1988, abstr. 19).

Poulson, D., and B. Sakaguchi. 1961. Nature of "sex-ratio" agent in *Drosophila*. Science 133:1489-1490.

Prins, R. A., and C. J. A. H. V. van den Vorstenbosch. 1975. Interrelationships between rumen microorganisms. Misc. Pap. Landbouwhogesch. Wageningen 11:15-24.

Pyle, L. E., L. N. Corcoran, B. G. Cocks, A. D. Bergemann, J. C. Whitley, and L. R. Finch. 1988. Pulsed-field electrophoresis indicates larger-than-expected sizes for mycoplasma genomes. Nucleic Acids Res. 16:6015.

Ranhand, J. M., I. Nur, D. L. Rose, J. G. Tully. 1987. *Spiroplasma* species share common DNA sequences among their viruses, plasmids and genomes. Ann. 'Inst. Pasteur Microbiol. 138:509-522.

Razin, S. 1975. The mycoplasma membrane. Prog. Surf. Membr. Sci. 9:257-312.

Razin, S. 1978. The mycoplasmas. Microbiol. Rev. 42:414-470.

Razin, S. 1982. Sterols in mycoplasma membranes. Pages 183-205 *in* S. Razin and S. Rottem (eds.), Current topics in membranes and transport. Volume 17: Membrane lipids of prokaryotes. Academic Press, Inc., New York.

Razin, S. 1985. Molecular biology and genetics of mycoplasmas (*Mollicutes*). Microbiol. Rev. 49:419-455.

Razin, S., and M. F. Barile (eds.). 1985. The mycoplasmas. Volume IV. Mycoplasma pathogenicity. Academic Press, Inc., Orlando.

Razin, S., and E. A. Freundt. 1984. Class I. Mollicutes. Pages 740-793 in N. R. Krieg and J. G. Holt (eds.), Bergey's manual of systematic bacteriology. Volume 1. Williams & Wilkins, Baltimore.

Razin, S., and J. G. Tully (eds.). 1983. Methods in mycoplasmology. Volume I. Mycoplasma characterization. Academic Press, Inc., New York.

Razin, S., S. Kutner, H. Efrati, and S. Rottem. 1980. Phospholipid and cholesterol uptake by mycoplasma cells and membranes. Biochim. Biophys. Acta 598:628-640.

Reeves, R. 1987. Metabolic energy supplied by PP_i. Pages 255-259 *in* A. Torriana-Gorini, F. G. Rothman, S. Silver, A. Wright, and E. Yagil (eds.), Phosphate metabolism and cellular regulation in microorganisms. American Society for Microbiology, Washington, D.C.

Reinards, R., J. Kubicki, and H.-D. Ohlenbusch. 1981. Purification and characterization of NADH oxidase from membranes of *Acholeplasma laidlawii*, a copper-containing iron-sulfur flavoprotein. Eur. J. Biochem. 120:329-337.

Roberton, A. M., and P. G. Glucina. 1982. Fructose 6-phosphate phosphorylation in *Bacteroides* species. J. Bacteriol. 150:1056-1060.

Robinson, I. M. 1973. Isolation and characterization of anaerobic mycoplasmas from the rumen. M.S. thesis, Iowa State University, Ames.

Robinson, I. M. 1979. Special features of anaeroplasmas. Pages 515-528 *in* M. F. Barile and S. Razin (eds.), The mycoplasmas. Volume 1. Cell biology. Academic Press, Inc., New York.

Robinson, I. M. 1984. Genus *Anaeroplasma*. Pages 787-790 *in* N. R. Krieg and J. G. Holt (eds.), Bergey's manual of systematic bacteriology. Volume 1. Williams & Wilkins, Baltimore.

Robinson, I. M., and M. J. Allison. 1975. Transfer of *Acholeplasma bactoclasticum* Robinson and Hungate to the genus *Anaeroplasma* (*Anaeroplasma bactoclasticum* [Robinson and Hungate] comb.nov.): Emended description of the species. Int. J. Syst. Bacteriol. 25:182-186.

Robinson, I. M., M. J. Allison, and P. A. Hartman. 1975. *Anaeroplasma abactoclasticum* gen.nov., sp.nov.: An obligately anaerobic mycoplasma from the rumen. Int. J. Syst. Bacteriol. 25:173-181.

Robinson, I. M. and E. A. Freundt. 1987. Proposal for an amended classification of anaerobic mollicutes. Int. J. Syst. Bacteriol. 37:78-81.

Robinson, I. M., and K. R. Rhoades. 1977. Serological relationships between strains of anaerobic mycoplasmas. Int. J. Syst. Bacteriol. 27:200-203.

Robinson, J. P. 1971. Studies on a *Mycoplasma* isolated from the bovine rumen. Ph.D. dissertation, University of California, Davis. (Diss. Abstr. Int. B Sci. Eng. 32:7196-B, 1972).

Robinson, J. P., and R. E. Hungate. 1973. *Acholeplasma bactoclasticum* sp. n., an anaerobic mycoplasma from the bovine rumen. Int. J. Syst. Bacteriol. 23:171-181.

Rodwell, A. W. 1969. Nutrition and metabolism of the mycoplasmas. Pages 413-449 *in* L. Hayflick (ed.), The mycoplasmatales and the L-phase of bacteria. Appleton-Century-Crofts, New York.

Rodwell, A. W. 1983. *Mycoplasma gallisepticum* requires exogenous phospholipid for growth. FEMS Microbiol. Lett. 17:265-268.

Rodwell, A. W., and A. Mitchell. 1979. Nutrition, growth, and reproduction. Pages 103-139 *in* M. F. Barile and S. Razin (eds.), The mycoplasmas. Volume I. Cell biology. Academic Press, Inc., New York.

Rodwell, A. W., and E. S. Rodwell. 1954*a*. The breakdown of carbohydrates by *Asterococcus mycoides*, the organism of bovine pleuropneumonia. Aust. J. Biol. Sci. 7:18-30.

Rodwell, A. W., and E. S. Rodwell. 1954*b*. The pathway for glucose oxidation by *Asterococcus mycoides,* the organism of bovine pleuropneumonia. Aust. J. Biol. Sci. 7:37-46.

Rogers, M. J., J. Simmons, R. T. Walker, W. G. Weisburg, C. R. Woese, R. S. Tanner, I. M. Robinson, D. A. Stahl, G. Olsen, R. H. Leach, and J. Maniloff. 1985. Construction of the mycoplasma evolutionary tree from 5S rRNA sequence data. Proc. Natl. Acad. Sci. USA 82:1160-1164.

Romano, N., D. Russo Alesi, R. La Licata, and G. Tolone. 1986. Effects of urea phosphate (sic), ammonium ions and pH on *Ureaplasma* ATP synthesis. Microbiologica (Bologna) 9:405-413.

Romano, N., G. Tolone, F. Ajello, and R. La Licata. 1980. Adenosine 5'-triphosphate synthesis induced by urea hydrolysis in *Ureaplasma urealyticum.* J. Bacteriol. 144:830-832.

Rose, C. E., and S. J. Pirt. 1981. Conversion of glucose to fatty acids and methane: Roles of two mycoplasmal agents. J. Bacteriol. 147:248-254.

Rottem, S., L. Adar, Z. Gross, Z. Ne'eman, and P. J. Davis. 1986. Incorporation and modification of exogenous phosphatidylcholines by mycoplasmas. J. Bacteriol. 167:299-304.

Rottem, S., and V. P. Cirillo. 1986. Transport in mycoplasmas. Methods Enzymol. 125:259-264.

Rottem, S., C. Linker, T. H. Wilson. 1981. Proton motive force across the membrane of *Mycoplasma gallisepticum* and its possible role in cell volume regulation. J. Bacteriol. 145:1299-1304.

Rottem, S., and O. Markowitz. 1979. Unusual positional distribution of fatty acids in phosphatidylglycerol of sterol-requiring mycoplasmas. FEBS Lett. 107:379-382.

Rottem, S., and S. Razin. 1967. Uptake and utilization of acetate by mycoplasma. J. Gen. Microbiol. 48:53-63.

Saglio, P. H. M., and R. F. Whitcomb. 1979. Diversity of wall-less prokaryotes in plant vascular tissue, fungi, and invertebrate animals. Pages 1-36 *in* R. F. Whitcomb and J. G. Tully (eds.), The mycoplasmas. Volume III. Plant and insect mycoplasmas. Academic Press, Inc., New York.

Saglio, P., M. Lhospital, D. LaFlèche, G. Dupont, J. M. Bové, J. G. Tully, and E. A. Freundt. 1973. *Spiroplasma citri* gen. and sp. n.: A mycoplasma-like organism associated with "stubborn" disease of citrus. Int. J. Syst. Bacteriol. 23:191-204.

Saglio, P. H. M., M. J. Daniels and A. Pradet. 1979. ATP and energy charge as criteria of growth and metabolic activity of mollicutes: Application to *Spiroplasma citri*. J. Gen. Microbiol. 110:13-20.

Salih, M. M., V. Simonsen, and H. Ernø. 1983. Electrophoretic analysis of isozymes of *Acholeplasma* species. Int. J. Syst. Bacteriol. 33:166-172.

Sanwal, B. D. 1970. Allosteric controls of amphibolic pathways in bacteria. Bacteriol. Rev. 34:20-39.

Sawyer, M. H., P. Baumann, and L. Baumann. 1977. Pathways of D-fructose and D-glucose catabolism in marine species of *Alcaligenes, Pseudomonas marina,* and *Alteromonas communis*. Arch. Microbiol. 112:169-172. [For current classification, see Int. J. Syst. Bacteriol. 33:793-802.].

Schummer, U., and H. G. Schiefer. 1987. Transmembrane proton-motive potential of *Spiroplasma floricola*. FEBS Lett. 224:71-82.

Sharp, J. T. 1954. L colonies from hemolytic streptococci: New technic in the study of L forms of bacteria. Proc. Soc. Exp. Biol. Med. 87:94-97.

Shepard, M. C. 1956. T-form colonies of pleuropneumonialike (sic) organisms. J. Bacteriol. 362-369.

Shepard, M. C., C. D. Lunceford, D. K. Ford, R. H. Purcell, D. Taylor-Robinson, S. Razin, and F. T. Black. 1974. *Ureaplasma urealyticum* gen. nov., sp. nov.: Proposed nomenclature for the human T (T-strain) mycoplasmas. Int. J. Syst. Bacteriol. 24:160-171.

Shepard, M. C., and G. K. Masover. 1979. Special features of ureaplasmas. Pages 451-494 *in* M. F. Barile and S. Razin (eds.), The mycoplasmas. Volume I. Cell biology. Academic Press, Inc., New York.

Shibata, K.-I., and T. Watanabe. 1986. Carboxypeptidase activity in human mycoplasmas. J. Bacteriol. 168:1045-1047.

Sladek, T. L. 1986. A hypothesis for the mechanism of mycoplasma evolution. J. Theor. Biol. 120:457-465.

Smart, J. B., and G. G. Pritchard. 1979. Regulation of pyruvate kinase from *Propionibacterium shermanii*. Arch. Microbiol. 122:281-288.

Smith, P. F. 1957. Conversion of citrulline to ornithine by pleuropneumonialike (sic) organisms. J. Bacteriol. 74:801-806.

Smith, P. F. 1971. The biology of mycoplasmas. Academic Press, Inc., New York.

Smith, P. F., and C. V. Henrikson. 1965. Comparative biosynthesis of mevalonic acid by *Mycoplasma*. J. Bacteriol. 89:146-153.

Smith, S. L., P. J. VanDemark, and J. Fabricant. 1963. Respiratory pathways in the *Mycoplasma*. I. Lactate oxidation by *Mycoplasma galliseptic*um. J. Bacteriol. 86:893-897.

Stanbridge, E. J., and M. E. Reff. 1979. The molecular biology of mycoplasmas. Pages 157-185 *in* M. F. Barile and S. Razin (eds.), The mycoplasmas. Volume I. Cell biology. Academic Press, Inc., New York.

Stephens, E. B., I. M. Robinson, and M. F. Barile. 1985. Nucleic acid relationships among the anaerobic mycoplasmas. J. Gen. Microbiol. 131:1223-1227.

Subcommittee on the taxonomy of *Mollicutes*. 1984. Minutes of interem meeting, 30 August and 6 September 1982, Tokyo, Japan. Int. J. Syst. Bacteriol. 34:361-365.

Subcommittee on the taxonomy of *Mycoplasmatales*. 1977. Minutes of interim meeting, 22 September 1976. London, United Kingdom. Int. J. Syst. Bacteriol. 27:392-394.

Taylor-Robinson, D., G. W. Czonka, and M. J. Prentice. 1977. Human intra-urethral inoculation of ureaplasmas. Q. J. Med. 46:309-326.

Taylor-Robinson, D., and R. N. Gourlay. 1984. Genus II. *Ureaplasma*, p. 770-775 *in* N. R. Krieg and J. G. Holt (eds.), Bergey's manual of systematic bacteriology. Volume 1. William & Wilkins, Baltomre.

Tourtellotte, M. E., and R. E. Jacobs. 1960. Physiological and serological comparisons of PPLO from various sources. Ann. N.Y. Acad. Sci. 79:521-530.

Tryon, V. V., and D. Pollack. 1984. Purine metabolism in *Acholeplasma laidlawii* B: Novel PP_i-dependent nucleoside kinase activity. J. Bacteriol. 159:265-270.

Tryon, V. V., and J. D. Pollack. 1985. Distinctions in *Mollicutes* purine metabolism: Pyrophosphate-dependent nucleoside kinase and dependence on guanylate salvage. Int. J. Syst. Bacteriol. 35:497-501.

Tully, J. G. 1984. Family II. *Acholeplasmataceae.* Pages 775-781 *in* N. R. Krieg and J. G. Holt (eds.), Bergey's manual of systematic bacteriology. Volume 1. Williams & Wilkins, Baltimore.

Tully, J. G. and S. Razin (eds.). 1983. Methods in mycoplasmology. Volume II. Diagnostic mycoplasmology. Academic Press, Inc., New York.

Tully, J. G., and R. F. Whitcomb (eds.). 1979. The mycoplasmas. Volume II. Human and animal mycoplasmas. Academic Press, Inc., New York.

Tully, J. G., D. L. Rose, P. Carle, J. M. Bové, K. J. Hackett, and R. F. Whitcomb. 1988. *Acholeplasma entomophilum* sp. nov. from gut contents of a wide range of host insects. Int. J. Syst. Bacteriol. 38:164-167.

Turunen, M., E. Parkkinen, J. Londesborough, and M. Korhola. 1987. Distinct forms of lactate dehydrogenase purified from ethanol- and lactate-producing cells of *Clostridium thermohydrosulfuricum.* J. Gen. Microbiol. 133:2865-2873.

VanDemark, P. J., and P. F. Smith. 1964*a*. Respiratory pathways in the *Mycoplasma.* II. Pathway of electron transport during oxidation of reduced nicotinamide adenine dinucleotide by *Mycoplasma hominis.* J. Bacteriol. 88:122-129.

VanDemark, P. J., and P. F. Smith. 1964*b*. Evidence for a tricarboxylic acid cycle in *Mycoplasma hominis.* J. Bacteriol. 88:1602-1607.

Wallace, D. C, and H. J. Morowitz. 1973. Genome size and evolution. Chromosoma (Berl.) 40:121-126.

Weisburg, W. G., J. G. Tully, D. L. Rose, J. P. Petzel, H. Oyaizu, D. Yang, L. Mandelco, J. Sechrest, T. G. Lawrence, J. van Etten, J. Maniloff, and C. R. Woese. 1989. A phylogenetic analysis of the mycoplasmas: Basis for their classification. J. Bacteriol., submitted for publication.

Whitcomb, R. F., and J. G. Tully (eds.). 1979. The mycoplasmas. Volume III. Plant and insect mycoplasmas. Academic Press, Inc., New York.

Whitcomb, R. F., and J. G. Tully. 1984. Family III. Spiroplasmataceae. Pages 781-787 *in* N. R. Krieg and J. G. Holt (eds.), Bergey's manual of systematic bacteriology. Volume 1. Williams & Wilkins, Baltimore.

Williams, M. V., and J. D. Pollack. 1985. Pyrimidine deoxyribonucleotide metabolism in members of the class *Mollicutes.* Int. J. Syst. Bacteriol. 35:227-230.

Williams, M. V., and J. D. Pollack. 1988. Uracil-DNA glycosylase activity: Relationship to proposed biased mutation pressure in the class *Mollicutes*. Pages 440-444 *in* R. E. Moses and W. C. Summers (eds.), DNA replication and mutagenesis. American Society for Microbiology, Washington, D.C.

Woese, C. R. 1987. Bacterial evolution. Microbiol. Rev. 51:221-271.

Woese, C. R., J. Maniloff, and L. B. Zablen. 1980. Phylogenetic analysis of the mycoplasmas. Proc. Natl. Acad. Sci. USA 77:494-498.

Woese, C. R., E. Stackebrandt, and W. Ludwig. 1985. What are mycoplasmas: The relationship of tempo and mode in bacterial evolution. J. Mol. Evol. 21:305-316.

Wood, H. G. 1985. Inorganic pyrophosphate and polyphosphates as sources of energy. Curr. Top. Cell. Regul. 26:355-369.

Wood, H. G., and N. H. Goss. 1985. Phosphorylation enzymes of the propionic acid bacteria and the roles of ATP, inorganic pyrophosphate, and polyphosphate. Proc. Natl. Acad. Sci. USA 82:312-315.

Yamada, T., and J. Carlsson. 1975. Glucose-6-phosphate-dependent pyruvate kinase in *Streptococcus mutans*. J. Bacteriol. 124:562-563.

ACKNOWLEDGMENTS

One goal I am still striving to achieve is to convey my thoughts by the written word as succinctly as possible. I have met with only limited success, as these acknowledgments demonstrate.

It is difficult to find the words to thank Paul A. Hartman, my co-major professor. He is an exemplary individual, both personally and scientifically. I hope that in the coming years his example will help me achieve some degree of the success that he has attained in his career. My thanks go to him for all that he has done for me. Milton J. Allison, also my co-major professor, deserves special recognition. I could always count on Milt for providing a different perspective on my research. I feel that I have profited from having had the opportunity to be associated with such an accomplished rumen microbiologist. I hope that Milt finds the anaerobic mollicutes as interesting as I have. Hopefully, he can find the time and resources to renew the studies of these bacteria at some level in the future.

A very special note of thanks is due Isadore M. Robinson. I am privileged to have been able to continue the studies started by Ike of these special bacteria. He has been a constant source of encouragement and help, from the benchtop to introductions to other mycoplasmologists. I remember with fondness the many times Ike and I got together to talk about the anaerobic mycoplasmas. Because we were are among the very few people to have ever worked with these bacteria, I suppose those

discussions might be considered "the International Meetings of Anaeroplasmology"!

I wish to thank the other members of my committee: Dr. Richard F. Ross, for his continuing interest in my project; Dr. John G. Holt, for the use of the files of the Bergey's Manual Trust; and Dr. Peter A. Pattee, for his unhestitating willingness to share equipment and resources. My thanks also go to Drs. Thomas Loynachan and Fred Williams for substituting at my final oral exam and for reading this thesis. I also thank Dr. Williams for the use of his French press.

I am also in debt to Dr. J. Dennis Pollack of Ohio State for providing an opportunity for a fruitful collaboration. His help, advice, and hospitality are greatly appreciated.

I'd like to thank my family and especially my Dad. I cannot express how much I value the understanding and support that he and my Mom gave me over my long educational career.

Two of my fellow students deserve special mention. My continuing association with Dr. John B. Luchansky is without a doubt one of the most fulfilling interactions of my career. John is a source of immeasurable help on many counts, and I cannot express how much I value his friendship. I'd also like to thank John for cultivating my tastes in Mexican food. I'm privileged to have met Andrew K. Benson here at Iowa State. Andy is one of the brightest people I know, and he is always a stimulus to remain sharp scientifically! I am happy to have him as a good friend. I am confident that these two individuals will have

successful and productive careers, and I look forward to watching them progress.

In addition, the following students, past and present, have my sincere gratitude. Dr. Charles W. Kaspar and his dogbears were of great help, personally and professionally. I thank Tim Freier for sharing with me both the wondrous world of anaerobes and the Green Death. I'd also like to thank Gina Balbi, Dr. David DeSantis of Ohio State, Carl Bortner, Dan Nieuwlandt, Vicky Carr-Brendel, Tracy J. Goist, Robyn Hottman, and Lillian McLeod. I have fond memories of our good times together.

Finally, I am in debt to the entire faculty of the Department of Microbiology at Iowa State insofar as each member, by his own striking example and conduct, has made clear to me how I wish to conduct myself personally and professionally.

This research was supported in part by the Iowa Agriculture and Home Economics Experiment Station, project 2678.

"Methought I heard a cry, 'Sleep no more!
Macbeth doth murder sleep' - the innocent sleep."

Macbeth, Act II, Scene II, by William Shakespeare.

APPENDIX A: CULTURE MEDIA FOR *ANAEROPLASMA* SPP. AND *ASTEROLEPLASMA ANAERTOBIUM*

This dissertation research required the routine cultivation and harvesting of the strictly anaerobic mollicutes. Rumen fluid-based media (Robinson, 1979) support good growth of these bacteria. However, rumen fluid-based media were deemed unsuitable for cultivation and harvesting of cells for physiological studies for a number of reasons. First, the collection and processing of rumen contents is laborious. Second, rumen fluid obtained at different times varies in its stimulatory properties. In addition, it was desired that the culture medium used for physiological studies not have an ingredient as complex and ill-defined as rumen fluid. Third, media made with some batches of rumen fluid had small amounts of precipitate after autoclaving (despite extensive processing of the rumen fluid). This precipitate may have interfered with the harvesting of cells and subsequent osmotic lysis and preparation of cell-free extracts.

After extensive variations were tried, two media were developed based on the formulation of MM-10 (Robinson, 1979). *Anaeroplasma* spp. were cultured in S-2 broth and *Asteroleplasma anaerobium* grew best in CAM broth (Table A1). These media had slight precipitates from the lipids, but they were minimal compared to other formulations.

The following observations were made about S-2 and CAM broths. Not all lots of Phytone, soluble starch, and carbohydrates supported good growth. If available, cell-culture tested products should be used and

several lots should be compared. Delivery of the lipids solution in a volume of less than 8 ml per liter of medium resulted in the medium being more opaque and less productive. Polyvinylpyrrolidone reduced the effects of some inhibitory lots of other ingredients. The media were sterilized in round-bottom flasks placed in containers to hold the flasks upright. The containers should allow free flow of the steam around the bottom of the flasks (i.e., wire baskets). If the flasks were placed in plastic beakers of slightly larger diameter than the flasks, the media were not sterile after 1 h at 121°C.

Media containing cottonseed peptones (Proflo, Traders Protein, Memphis, TN) or soybean lipids (#652 229; Boehringer Mannheim, Indianapolis, IN) were substantially more stimulatory than the standard formulation of S-2 broth (Table A1), but their addition resulted in a greater amount of precipitate.

Finally, in the earlier 1980s, Corning and Kimble redesigned their lines of round-bottom flasks, increasing the diameters of the necks. For example, the 500-ml flask was changed from a #4-size stopper to #6, and the 1000-ml flask was changed from a #6 stopper to #8. These larger-neck flasks have less mechanical strength, and they frequently broke in the autoclave. Therefore, custom-made flasks with the narrower necks were obtained at moderate prices from Lab Glass, Inc., Vineland, NJ.

Table A1. Culture media for strictly anaerobic mollicutes

Ingredient	S-2 Broth[a]	CAM Broth[a]
BBL Phytone [or Deltown Chemurgic papaic digest of soy]	2.0 g	-
Difco brain heart infusion	2.0 g	-
Difco Casamino acids	-	6.0
Difco Soytone	1.0 g	-
Difco malt extract	1.5 g	1.5 g
Yeast extract	1.0 g	1.0 g
Maltose	1.0 g	1.0 g
Glucose	0.5 g	0.5g
Difco soluble starch	2.5 g	2.5 g
Polyvinylpyrrolidone (Sigma PVP-40)	10.0 g	10.0 g
H_2O	875 ml	875 ml

[a]Both media are supplemented with minerals, volatile fatty acids, resazurin, and cysteine hydrochloride as specified in Appendix C, Table C1. The pH is adjusted to 7.2 with approximately 6.25 ml of 7.5 *M* NaOH. The media are prepared anaerobically under CO_2 by the modified Hungate technique. After sterilization, the media are supplemented with 94 ml of 8% Na_2CO_3 (sterile and under CO_2) and 8 ml of an ethanolic solution of cholesterol (2.5 mg/ml, analytical grade) and soybean lecithin (22.5 mg/ml of Calbiochem 429415; other preparations were not satisfactory). The cholesterol is dissolved by warming at 55°C in a stoppered tube. The lipids solution is sterilized by filtration (Millipore SLGV025LS); final concentrations of the lipids in the media are 20 µg of cholesterol/ml and 180 µg of lecithin/ml. The medium is then sparged with CO_2 until the resazurin is once again colorless. See text for discussion.

APPENDIX B:
ENZYMIC ACTIVITIES OF STRICTLY ANAEROBIC MOLLICUTES DETERMINED WITH API ZYM GALLERIES

Table B1. Enzymic activities of strictly anaerobic mollicutes determined with API ZYM substrate galleries

Enzyme	Strain[a] JR^T	Strain 7LA^T	Strain 5LA	Strain A-2^T	Strain 161^T
Peptidase gallery I:					
L-Tyr arylamidase	-[b]	-	-	-	-
L-pyrrolidone arylamidase	1+	-	-	-	-
L-Phe arylamidase	1+	±	±	-	-
L-Lys arylamidase	1+	3+	5+	1+	3+
L-Hydroxyproline arylamidase	3+	5+	5+	3+	5+
L-His arylamidase	1+	1+	3+	1+	2+
Gly arylamidase	1+	±	±	-	-
L-Asp arylamidase	-	-	-	-	-
L-Arg arylamidase	1+	2+	3+	-	2+
L-Ala arylamidase	2+	2+	3+	2+	-
Peptidase gallery II:					
Γ-Glutamyltransferase	-	-	-	-	-
N-Benzoyl-leu arylamidase	-	-	-	-	-

[a]Strain JR^T = *Anaeroplasma bactoclasticum;* strains 7LA^T and 5LA = *An. intermedium;* strain A-2 = *An. varium;* strain 161^T = *Asteroleplasma anaerobium.* Cells were harvested from 800 ml of S-2 broth (Appendix A), washed in EW buffer (Section I), and resuspended in 0.85% NaCl

[b]Except for the Osidase gallery, all reactions were scored on a scale of 1+ to 5+ (5+ being the most intensely positive) by using a scoring chart provided with the galleries. Negative reactions are reported as "-", and equivocal reactions are reported as "±". Reactions for the Osidase gallery are reported as positive (+), negative (-), or equivocal (±).

Table B1 (continued)

Enzyme	Strain JR^T	Strain 7LA^T	Strain 5LA	Strain A-2^T	Strain 161^T
S-Benzyl-cys arylamidase	-	±	±	-	-
Met arylamidase	1+	1+	2+	1+	±
Gly-gly arylamidase	3+	1+	1+	2+	-
Gly-phe arylamidase	1+	±	1+	1+	-
Gly-pro arylamidase	±	3+	2+	1+	3+
Leu-gly arylamidase	2+	±	1+	1+	-
L-Ser-tyr arylamidase	1+	-	±	±	±
Peptidase gallery III:					
N-CBZ-arg-4-methoxy arylamidase	-	-	-	-	-
L-Gln arylamidase	2+	2+	3+	1+	1+
α-L-Glu arylamidase	-	-	-	-	-
L-Ile arylamidase	±	±	±	-	-
L-Orn arylamidase	1+	1+	2+	±	1+
L-Pro arylamidase	3+	5+	5+	4+	5+
L-Ser arylamidase	1+	1+	2+	1+	-
L-Thr arylamidase	-	-	-	-	-
L-Trp arylamidase	2+	1+	2+	-	-
N-CBZ-gly-gly-arg arylamidase	-	-	-	-	-
Peptidase gallery IV:					
β-Ala arylamidase	-	-	-	-	-

Table B1 (continued)

Enzyme	Strain JR^T	Strain 7LA^T	Strain 5LA	Strain A-2^T	Strain 161^T
L-Ala-L-arg arylamidase	1+	1+	2+	1+	-
L-Ala-L-phe-L-pro arylamidase	1+	1+	2+	1+	±
L-Ala-L-phe-L-pro-L-ala arylamidase	2+	-	-	±	-
L-arg-L-arg arylamidase	1+	-	1+	±	-
α-L-Asp-L-ala arylamidase	3+	1+	3+	2+	-
α-L-Asp-L-arg arylamidase	±	-	±	1+	-
α-L-Glu-α-L-glu arylamidase	-	-	-	-	-
α-L-Glu-L-his arylamidase	±	±	1+	±	-
Gly-L-ala arylamidase	2+	1+	2+	1+	-
Peptidase gallery V:					
Gly-L-arg arylamidase	1+	±	1+	±	-
Gly-L-trp arylamidase	1+	-	1+	±	-
L-His-L-leu-L-his arylamidase	±	-	-	±	1+
L-His-L-ser arylamidase	±	±	-	±	-
L-Leu-L-ala arylamidase	2+	1+	2+	1+	-
L-Leu-L-leu-L-valyl-L-tyr-L-ser arylamidase	-	-	-	±	-
L-Lys-L-ala arylamidase	2+	-	1+	±	-
L-Lys-L-lys arylamidase	±	-	±	±	-
L-Phe-L-arg arylamidase	±	-	1+	±	±
L-Phe-L-pro arylamidase	1+	3+	3+	1+	4+

Table B1 (continued)

Enzyme	Strain JR^T	Strain 7LA^T	Strain 5LA	Strain A-2^T	Strain 161^T
Peptidase gallery VI:					
L-Phe-L-pro-L-ala arylamidase	2+	-	-	±	2+
L-Pro-L-arg arylamidase	1+	-	1+	±	-
L-Ser-L-arg arylamidase	2+	±	2+	1+	-
L-Valyl-L-tyr-L-ser arylamidase	±	-	-	-	-
N-Benzoyl-L-ala-4-methoxy arylamidase	-	-	-	-	-
N-CBZ-arg-4-methoxy arylamidase	-	-	-	-	-
N-Acetyl-gly-L-lys arylamidase	-	-	-	-	-
L-His-L-phe arylamidase	1+	-	-	±	-
L-Lys-L-ser-4-methoxy arylamidase	±	-	-	±	-
Esterase gallery:					
Butyrate	1+	5+	5+	1+	5+
Valerate	2+	5+	5+	2+	5+
Caproate	3+	5+	5+	3+	5+
Caprylate	3+	5+	5+	3+	5+
Nonanoate	3+	5+	4+	3+	5+
Caprate	3+	2+	5+	3+	3+
Laurate	±	1+	3+	2+	1+
Myristate	-	-	2+	±	-

Table B1 (continued)

Enzyme	Strain JR^T	Strain 7LA^T	Strain 5LA	Strain A-2^T	Strain 161^T
Palmitate	-	-	-	-	-
Stearate	±	2+	2+	±	2+
"Osidase" (Glycosidase) gallery:					
α-D-Galactosidase	+	+	+	+	+
β-D-Galactosidase	+	+	+	+	±
Phospho-β-D-galactosidase	-	-	-	-	-
α-L-Arabinosidase	-	-	-	-	-
α-D-Glucosidase	+	+	±	+	±
β-D-Glucosidase	+	-	-	±	+
β-D-Galacturonohydrolase	-	-	-	-	-
β-D-Glucuronidase	-	-	-	-	+
α-Maltosidase	+	+	+	+	±
β-Maltosidase	-	-	-	-	-
N-Acetyl-α-D-glucosaminidase	-	-	-	-	-
N-Acetyl-β-D-glucosaminidase	-	-	-	-	-
α-L-Fucosidase	-	-	-	-	-
β-D-Fucosidase	+	-	+	±	+
β-L-Fucosidase	-	-	-	-	-
β-D-Lactosidase	+	-	-	±	-
α-D-Mannosidase	-	-	-	-	-
β-D-Mannosidase	-	-	-	-	-

Table B1 (continued)

Enzyme	Strain JR^T	Strain 7LA^T	Strain 5LA	Strain A-2^T	Strain 161^T
α-D-Xylosidase	-	-	-	-	-
β-D-Xylosidase	+	-	-	±	+
Standard API ZYM gallery:					
Alkaline phosphatase	2+	-	-	1+	1+
C4 esterase (butyrate)	1+	1+	3+	2+	4+
C8 esterase (caprylate)	3+	2+	1+	2+	4+
Lipase (myristate)	±	±	-	±	±
Leu aminopeptidase	2+	1+	2+	±	1+
Val aminopepetidase	1+	-	-	-	-
Cys aminopeptidase	-	-	-	-	-
Trypsin	-	-	-	-	±
Chymotrypsin	-	-	-	-	-
Acid phosphatase	3+	1+	±	1+	3+
Phosphohydrolase	-	±	-	-	-
α-Galactosidase	3+	1+	±	1+	2+
β-Galactosidase	4+	±	-	3+	5+
β-Glucuronidase	-	-	-	-	5+
α-Glucosidase	4+	3+	4+	3+	3+
β-Glucosidase	1+	-	-	1+	4+
N-Acetyl-β-glucosaminidase	±	-	-	-	-
α-Mannosidase	-	-	-	-	-
α-Fucosidase	-	-	-	-	-

APPENDIX C: STIMULATION OF GROWTH OF *ANAEROPLASMA* SPP. BY VARIOUS CARBON SOURCES

Although all *Anaeroplasma* spp. are stimulated by soluble starch, initial reports indicated that some strains were not stimulated by glucose or maltose (Robinson, 1979; Robinson and Allison, 1975). Therefore, as a prelude to the studies of carbohydrate metabolism in the present investigation, three *Anaeroplasma* spp. were tested for stimulation of growth by various mono-, di-, and oligosaccharides, and carbohydrate polymers.

Anaeroplasmas can be cultivated in completely defined media; peptones and yeast extract are replaced by amino acids and/or vitamins (Robinson, 1979). In the present investigation, growth of anaeroplasmas in a similar medium was poor, and the medium was easily oxidized because of the absence of a variety of oxygen-scavenging compounds that are present in peptones and yeast extract. Therefore, the carbon-source studies were performed with a semi-defined medium based on a modification of medium D of Robinson (1979) (Table C1).

The data in Table C2 show that all three *Anaeroplasma* spp. were stimulated by soluble starch and glycogen as well as glucose and oligosaccharides (maltose, maltotriose, maltoheptaose, α-cyclodextrin, and β-cyclodextrin) with the α-1,4 glucosidic linkage. The stimulatory properties of the other carbon sources were mixed.

Table C1. Semi-defined medium for the determination of stimulatory properties of various carbon sources on *Anaeroplasma* spp.

Ingredient	Amount
Carbon source[a]	0.2 g
Mineral solution 1[b]	3.75 ml
Mineral solution 2[c]	3.75 ml
Yeast extract	0.1 g
Volatile fatty acids[d]	0.31 ml
Cysteine·HCl·H_2O	0.05 g
Resazurin solution[e]	0.1 ml
Lipids solution[f]	3.0 ml
Na_2CO_3 solution[g]	5.0 ml
H_2O	100.0 ml

[a]Except for starch and glycogen, carbon sources were filter-sterilized and added to the medium after autoclaving. The medium was prereduced and anaerobically sterilized under CO_2 as described by Robinson (1979) and in Appendix A (Table A1).

[b]Mineral solution 1 (g/l): K_2HPO_4, 6.0.

[c]Mineral solution 2 (g/l): KH_2PO_4, 6.0; NaCl, 12.0; Na_2SO_4, 6.5; $CaCl_2 \cdot 2H_2O$, 1.2; $MgCl_2$, 0.61.

[d]Volatile fatty acid solution (ml of individual acid/l of solution): acetic, 548; propionic, 194; *n*-butyric, 129; *n*-valeric, 32; isovaleric, 32; isobutyric, 32; DL-α-methylbutyric, 32.

[e]Resazurin solution, 1.0 g/l.

[f]Lipids solution: 10 mg of cholesterol (analytical grade) was placed in 5 ml of ethanol; the tube was stoppered and placed at 55°C until the cholesterol was dissolved. Fifty mg of phosphatidylcholine in chloroform (Sigma P-6263) were flushed with N_2 until the chloroform evaporated; the lipid was taken up in 2 ml of ethanol, and 1 ml of the cholesterol solution was added to make the complete lipids solution.

[g]Na_2CO_3, 8.0 g/l.

Table C2. Stimulatory properties of various carbon sources on the growth of three *Anaeroplasma* spp.[a]

Carbon source	*Anaeroplasma bactoclasticum* JR^T	*Anaeroplasma intermedium* 5LA	*Anaeroplasma varium* A-2^T
Glycerol	-[b]	-	-
Glucose	4+	4+	1+
Glucose-1-phosphate	2+	-	1+
Glucose-6-phosphate	1+	-	1+
Fructose	1+	-	-
Maltose	4+	3+	4+
Cellobiose	1+	-	3+
Sucrose	1+	-	1+
Isomaltose	-	2+	-
Maltotriose	4+	2+	4+
Maltoheptaose	4+	2+	4+
α-Cyclodextrin	1+	3+	2+
β-Cyclodextrin	1+	3+	2+
Soluble starch	4+	3+	3+
Glycogen	3+	3+	4+

[a]Each strain was passed three times (48-h incubation/passage) through the semi-defined medium (Table C1) supplemented to 0.2% with the indicated carbon source.

[b]Results are based on viable counts of the third passage after 48-h incubation. Viable counts for each carbon source were compared with counts of basal medium without supplementary carbon source: - = no stimulation; 1+ = 2-10x viable count of basal medium; 2+ = 10-100x viable count of basal medium; 3+ = 100-1000x viable count of basal medium; 4+ = 1000-6000x viable count of basal medium.